THE RELATION OF BACTERIOLYSIS TO PROTEOLYSIS.

Studies on Ferment Action. XVI.*

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The relation of the proteolytic ferments of serum to immunity reactions, particularly to bacteriolysis, has interested several investigators, and in view of the recent work of Abderhalden and his collaborators it merits attention quite apart from a theoretical consideration. The study of possible proteolytic cleavage during bacteriolysis, has, because of the manifest technical difficulties entailed by the accurate determination of nitrogen in the small amounts available, been limited to rather general statements, based, in most instances, on experiments carried out with the Sörensen method. This method, being an index of the total cleavage of the protein molecule, gives no information of the change from coagulable to non-coagulable forms, and is therefore of use in estimating peptolytic or ereptic enzyme activity rather than proteolytic changes. For a like reason it represents the splitting of the protein bodies to non-toxic fragments in contradistinction to the splitting of the whole non-toxic protein molecule to the higher non-coagulable but toxic forms (proteoses). Since the microchemical methods devised by Bang (1) and Folin (2) were described there are available no experiments dealing with this subject, and we have therefore undertaken the following experiments using the Folin method, which lends itself admirably to work of this kind.

The bactericidal and bacteriolytic property of fresh serum due to the action of complement and amboceptor has been assumed, more particularly by French and German writers, to be enzymotic in character and in the nature of a protease. There is, however, no experimental basis for this idea; indeed the fact that complement

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action is not identical with a protease action can be demonstrated in a very simple experiment. When chloroform is added to fresh serum and thoroughly mixed, the complement is very rapidly destroyed; the serum protease, however, first becomes active under these conditions (Delezenne and Pozerski (3)). The activation under the influence of chloroform or other lipoid solvents is due to the fact that the antiferment is removed in this way. There should therefore be no confusion as to the non-identity of these serum constituents. If complement activity is enzymotic in character, we should rather seek to identify it with the lipases or allied enzymes. Thus the objection made that complement acts in direct proportion to the concentration, and is therefore unlike other ferments, fails in view of the fact that serum esterase acts in a like manner (Loevenhart (4)). And the ease with which lipoid solvents inactivate the complement finds an analogy in plant lipases which are either destroyed by lipoidal solvents or are in such intimate relation to the lipoids that they are taken up with them into the solvent. The substrate offered for complement action—red blood cells, animal cells, bacteria—is lipoidal in nature, the potentially lipoidal character of the limiting membranes of the corpuscles and animal cells being well established and rendered more than probable for bacterial cells. The fact that powerful proteolytic enzymes are without effect on such substrata is almost conclusive evidence that the surfaces of these substrata are not protein in nature. An observation of Metchnikoff (5) is significant in this connection. Metchnikoff, noting that the intestinal tract of certain insects (moths) was free from bacteria, an exception to the condition found in other insects, concluded that these insects must secrete a powerful fat-splitting enzyme to enable them to utilize waxes and fats readily, and surmised some connection between the character of this intestinal secretion and the freedom from bacteria.

EXPERIMENTAL.

The experiments, designed to determine the amount of proteolysis occurring during bacteriolysis, were carried out in the following manner. The organisms used, *Bacillus coli, Bacillus typhosus,* and staphylococci, were grown on agar in large bottles. They were

washed repeatedly with salt solution and used as fresh emulsions, or dried at low temperature and made up in a 1 per cent. emulsion as used. In either case the total nitrogen and the total non-coagulable nitrogen per cubic centimeter of the emulsion were determined for several samples. Immune sera were obtained by bleeding rabbits when the blood contained the greatest concentration of immune bodies. The immune sera so obtained were kept without preservatives on ice and were inactive. Fresh guinea pig serum was used as complement.

One cubic centimeter of the bacterial emulsion was measured into small centrifuge tubes, the various sera were then added, and digestion was permitted at 37° C. under toluol for varying periods of time. The tubes were then thoroughly centrifuged until the supernatant fluid was clear. The fluid was carefully pipetted from the bacteria, these were washed once with salt solution, and the fluid was added to that already withdrawn. The total nitrogen of the bacterial rest was then determined and also the total non-coagulable nitrogen of the supernatant fluid. The difference between this latter figure and the amount of total non-coagulable nitrogen in the serum controls gives the amount of nitrogen which has autolyzed, *i. e.*, the bacterial nitrogen digestion. The difference between the total nitrogen of the bacteria originally introduced and the amount recovered indicates the amount of bacteriolysis.

In using the relatively large amounts of organisms necessitated by the character of the work, the amount of serum is by comparison small, but the bacteriolytic effect is in most cases quite evident by the greater loss of bacterial nitrogen in the tubes containing the immune serum and complement.

The work has been carried out in duplicate and in some instances four or more determinations were made on corresponding mixtures. For the sake of brevity the protocols have been condensed and show only single determinations. The variations were seldom greater than a few hundredths of a milligram. The figures indicated in the protocols express nitrogen in milligrams.

In numerous experiments the bacterial rests remaining after serum treatment were digested by trypsin after determinations on similar mixtures showed the amount of substrate remaining. The

PROTOCOL I.

Tube No.	Action of immune serum and complement on bacteria and relation of resulting lysis to proteolysis.									Action of trypsin on bacteria following immune serum and complement action.					
	Typhoid bacilli.	Colon bacilli.	Colon immune serum.	Complement.	Bacterial nitrogen recovered.	Loss in bacterial nitrogen.	Total non-coagulable nitrogen in supernatant fluid.	Gain in non-coagulable nitrogen in supernatant fluid.		Typhoid bacilli.	Colon bacilli.	Trypsin.	Bacterial nitrogen (substrate).	Nitrogen digestion.	Digestion.
1	1.0 c.c.	Total nitrogen = 1.65 mg. per c.c.					Total non-coagulable nitrogen = 0.25 mg. per c.c.			.....	.....	.....	.....	.....	.....
2	.....	1.0 c.c.	Total nitrogen = 2.80 mg. per c.c.				Total non-coagulable nitrogen = 0.38 mg. per c.c.			.....	.....	.....	.....	.....	.....
3	1.0 c.c.	.....	.....	.....	1.4 mg.	0.25 mg.	0.2 mg.	.....	Autolysis in salt solution	.....	.....	.....	.....	.....	.....
4	.....	1.0 c.c.	.....	.....	2.46 mg.	0.34 mg.	0.3 mg.	.....							
5	1.0 c.c.	.....	.....	.....	1.51 mg.	0.14 mg.	0.2 mg.	Total nitrogen in supernatant fluid		1.0 c.c.	.....	0.1 c.c.	1.45 mg.	0.78 mg.	49%
6	.....	1.0 c.c.	.....	.....	2.34 mg.	0.46 mg.	0.53 mg.			.....	1.0 c.c.	0.1 c.c.	2.4 mg.	0.75 mg.	31%
7	1.0 c.c.	.....	1.0 c.c.	2.0 c.c.	1.25 mg.	0.40 mg.	0.87(−0.625) mg.	0.24 mg.	Typhoid bacilli, colon immune serum complement	1.0 c.c.	.....	0.1 c.c.	1.25 mg.	0.48 mg.	38%
8	1.0 c.c.	.....	0.1 c.c.	2.0 c.c.	1.25 mg.	0.40 mg.	0.66(−0.437) mg.	0.22 mg.		1.0 c.c.	.....	0.1 c.c.	1.25 mg.	0.50 mg.	40%
9	.....	1.0 c.c.	1.0 c.c.	2.0 c.c.	2.10 mg.	0.7 mg.	1.0 (−0.625) mg.	0.375 mg.	Colon bacilli, colon immune serum complement	.....	1.0 c.c.	0.1 c.c.	2.10 mg.	0.77 mg.	34%
10	.....	1.0 c.c.	0.1 c.c.	2.0 c.c.	2.10 mg.	0.7 mg.	0.8 (−0.437) mg.	0.363 mg.		.....	1.0 c.c.	0.1 c.c.	2.10 mg.	0.508 mg.	24%
11	1.0 c.c.	.....	.....	2.0 c.c.	1.1 mg.	0.55 mg.	0.61(−0.417) mg.	0.2 mg.	Complement	1.0 c.c.	.....	0.1 c.c.	1.1 mg.	0.45 mg.	40%
12	.....	1.0 c.c.	.....	2.0 c.c.	2.34 mg.	0.46 mg.	0.8 (−0.417) mg.	0.383 mg.		.....	1.0 c.c.	0.1 c.c.	2.34 mg.	0.57 mg.	24%
13	1.0 c.c.	.....	1.0 c.c.	.....	1.2 mg.	0.45 mg.	0.5 (−0.207) mg.	0.293 mg.	Immune serum	1.0 c.c.	.....	0.1 c.c.	1.2 mg.	0.55 mg.	45%
14	.....	1.0 c.c.	1.0 c.c.	.....	2.4 mg.	0.4 mg.	0.56(−0.207) mg.	0.353 mg.		.....	1.0 c.c.	0.1 c.c.	2.4 mg.	1.0 mg.	40%
15	.....	.....	1.0 c.c.	.....	.....	.....	0.207 mg.	.....	Non-coagulable nitrogen in serum controls	.....	.....	.....	.....	.....	.....
16	.....	.....	.....	2.0 c.c.	.....	.....	0.417 mg.	.....		.....	.....	.....	.....	.....	.....

per cent. of digestion was determined by carefully acidifying and boiling and filtering through kaolinized hard paper filters to remove the coagulated protein. The solution of trypsin used was prepared each time from a dried preparation and used in such dilution that 0.1 of a cubic centimeter of the solution would digest two cubic centimeters of a 1 per cent. casein solution in two hours. Protocol I will serve as an example of such an experiment.

PROTOCOL I—A.

Autolysis in Physiological Salt Solution. Relation of Solution of Bacteria to Autolysis.

Tube No.	Typhoid bacilli.	Colon bacilli.	Total nitrogen in supernatant fluid after		Total non-coagulable nitrogen in supernatant fluid after		
			24 hrs.	48 hrs.	24 hrs.	48 hrs.	
1	1.0 c.c.	.....	0.37 mg.	.....	.....	.....	Solution.
2	1.0 c.c.	.....	.....	0.6 mg.	.....	.....	
3	1.0 c.c.	.....	.....	.....	0.2 mg.	.....	Autolysis.
4	1.0 c.c.	.....	.....	.....	.....	0.2 mg.	
5	.....	1.0 c.c.	0.57 mg.	.....	.....	.....	Solution.
6	.....	1.0 c.c.	.....	0.8 mg.	.....	.....	
7	.....	1.0 c.c.	.....	.....	0.35 mg.	.....	Autolysis.
8	.....	1.0 c.c.	.....	.....	.....	0.5 mg.	

Fresh typhoid and colon bacilli were used. The typhoid emulsion contained 1.65 milligrams of total nitrogen and 0.25 of a milligram of total non-coagulable nitrogen per cubic centimeter. The colon emulsion contained 2.80 milligrams of total nitrogen and 0.38 of a milligram of total non-coagulable nitrogen per cubic centimeter. Bacteria were permitted to autolyze in salt solution over night (tubes 3 to 6); with immune colon serum and fresh complement (tubes 7 to 10); with complement alone (tubes 11 and 12); and with immune colon serum alone (tubes 13 and 14). The total non-coagulable nitrogen in the sera used is shown in tubes 14 and 15, and these amounts, viz., $0.417 + 0.207 = 0.624$ of a milligram, $0.417 + 0.02 = 0.437$ of a milligram are, of course, to be deducted from the digestion mixtures. A parallel series of tubes was prepared, serum action permitted for an equal length of time, the bacteria washed and subjected to tryptic digestion for six hours.

It is apparent in observing the results obtained that the greatest

loss of bacterial nitrogen has occurred in the mixtures with colon bacilli, colon immune serum, and complement, indicating a marked lysis of these bacteria. There is, however, no corresponding increase in non-coagulable nitrogen obtained in the supernatant fluid, the amount so recovered in the 1.0 and 0.1 cubic centimeter dilutions of immune serum being respectively 0.375 of a milligram and 0.363 of a milligram, corresponding very well with the amount of non-coagulable nitrogen introduced originally with the bacteria, 0.38 of a milligram, and representing only a trace more than the amount recovered in the salt solution control (tube 4). There is obviously no relation here between the amount of lysis and the actual proteolysis. It is interesting to note that the greatest lysis of typhoid bacilli occurred in the mixture containing complement alone, although here, too, there is no corresponding increase in non-coagulable nitrogen. When the bacterial residues are digested by trypsin the percentage of digestion is found to be considerably less in all the tubes treated with serum than in the untreated controls (tubes 5 and 6), with the exception of the bacteria treated with immune serum (tubes 9 and 14). We have previously shown (6) that bacteria treated with normal serum become more resistant to tryptic digestion, because of their adsorption of serum antitrypsin.

In protocol I-A is shown the rate of solution and of autolysis of

PROTOCOL II.

Action of Typhoid Immune Serum and Complement on Typhoid Bacilli. No Demonstrable Proteolysis.

Tube No.	Typhoid bacilli.	Immune serum.	Complement.	Bacterial nitrogen recovered.	Loss.	Total nitrogen in supernatant fluid.	Gain in total nitrogen.	Total non-coagulable nitrogen in supernatant fluid.	Gain in non-coagulable nitrogen.
1	1.0 c.c.	Total nitrogen = 1.1 mg., total non-coagulable nitrogen = 0.16 mg. per c.c.							
2	1.0 c.c.	Autolysis in sodium carbonate solution		0.66 mg.	0.44 mg.	0.43 mg.	.....	0.17 mg.	.....
3	1.0 c.c.	0.1 c.c.	1.0 c.c.	0.83 mg.	0.27 mg.	7 (−6.7) mg.	0.3 mg.	.....	.....
4	1.0 c.c.	0.1 c.c.	1.0 c.c.	0.83 mg.	0.27 mg.	.....	.....	0.55 (−0.39) mg.	0.16 mg.
5	.....	0.1 c.c.	1.0 c.c.	.....	.....	6.7 mg.	.....	.....	.....
6	.....	0.1 c.c.	1.0 c.c.	.....	.....	.....	.....	0.39 mg.	.....

these organisms when kept in salt solution at 37° C. under toluol. While solution has occurred in both cases, the autolysis is not equally rapid; indeed it did not occur at all in the typhoid emulsion in this case.

In protocol II a typhoid system was used, with fresh typhoid bacilli and typhoid immune serum. The total nitrogen per cubic centimeter was 1.1 milligrams; the total non-coagulable nitrogen was 0.16 of a milligram per cubic centimeter. Mixtures were made as usual, the reaction in this case being made distinctly alkaline to phenolphthalein, the control being placed in a sodium carbonate solution of equal alkalinity. The control lost 0.44 of a milligram of bacterial nitrogen, 0.43 of a milligram being recovered from the supernatant fluid, of which 0.17 of a milligram was non-coagulable, indicating a lysis without proteolysis in the control. It will be recalled that autolytic enzymes in general act best in a slightly acid medium. In the immune serum (tubes 3 and 4) there has been less lysis than in the alkaline control (loss of 0.27 of a milligram), and while this amount was recovered in the increase in total nitrogen in the supernatant fluid (0.3 of a milligram), the non-coagulable nitrogen had not increased (0.16 of a milligram). It is quite apparent that the solution of the bacteria, due in one case to the alkalinity of the fluid (tube 2), in the other to the lytic effect of the serum complement and immune body (tubes 3 and 4), is not accompanied by proteolysis.

If we permit the action of the immune serum and complement on the bacteria for a shorter period of time (4 hours at 37° C.),

PROTOCOL III.

Action of Typhoid Immune Serum and Complement on Typhoid Bacteria. Absence of Proteolysis after 4 Hours' Incubation.

Tube No.	Typhoid bacilli.	Typhoid immune serum.	Complement.		Bacterial nitrogen recovered.	Loss.	Gain in non-coagulable nitrogen.
1	1.0 c.c.	Total nitrogen = 1.42 mg., total non-coagulable nitrogen = 0.2 mg. per c.c.					
2	1.0 c.c.	0.0 c.c.	0.0 c.c.	Autolysis	1.2 mg.	0.22 mg.	0.15 mg.
3	1.0 c.c.	0.0 c.c.	1.0 c.c.	.....	1.2 mg.	0.22 mg.	0.135 mg.
4	1.0 c.c.	1.0 c.c.	1.0 c.c.	.....	1.28 mg.	0.14 mg.	0.07 mg.
5	1.0 c.c.	0.1 c.c.	1.0 c.c.	.....	1.25 mg.	0.17 mg.	0.13 mg.
6	1.0 c.c.	0.01 c.c.	1.0 c.c.	.....	1.28 mg.	0.14 mg.	0.125 mg.

which is ample for the bactericidal effect of the serum even if not for actual bacteriolysis, there is practically no loss in bacterial nitrogen and no increase in non-coagulable nitrogen. As a matter of fact, there is much less splitting in the tube containing the greatest amount of antibody (tube 4, protocol III).

The fact that complement alone has no appreciable proteolytic effect even when bactericidal is shown clearly in protocol IV, in

PROTOCOL IV.

Effect of Complement on Colon Bacilli. No Resulting Proteolysis.

Tube No.	Colon bacilli.	Complement.	Reaction.	Bacterial nitrogen recovered.	Loss.	Total nitrogen recovered in supernatant fluid.	Total non-coagulable nitrogen in supernatant fluid.	Gain in non-coagulable nitrogen.
1	1.0 c.c.	Total nitrogen = 0.9 mg. per c.c.				.....	.....	.....
2	1.0 c.c.	.....	Acid	0.39 mg.	0.51 mg.	0.5 mg.	.....	0.17 mg.
3	1.0 c.c.	.....	Alkaline	0.39 mg.	0.51 mg.	0.5 mg.	.....	0.15 mg.
4	1.0 c.c.	1.0 c.c.	Acid	0.56 mg.	0.34 mg.	.....	0.45(−0.23) mg.	0.22 mg.
5	1.0 c.c.	1.0 c.c.	Alkaline	0.44 mg.	0.44 mg.	.....	0.3 (−0.23) mg.	0.07 mg.
6	.....	1.0 c.c.	.....	.....	.....	.....	0.23 mg.	.....

which dried colon bacilli were used. Dried organisms are, as a rule, more easily digested by trypsin than freshly killed organisms, possibly due to alterations in the physical state of the external limiting membrane of the organisms. The bacillary emulsion contained 0.9 of a milligram of total nitrogen per cubic centimeter. When permitted to autolyze in salt solution, both acid and alkaline in reaction, the solution of the organisms was quite marked, only 0.39 of a milligram remaining after twenty-four hours. The total dissolved nitrogen recovered from the supernatant fluid was 0.5 of a milligram, of which 0.17 of a milligram was non-coagulable. When treated with fresh guinea pig complement the lysis was not so marked, being greatest in the tubes with an alkaline reaction; the proteolysis, on the other hand, was greatest in an acid reaction. The increase of 0.05 of a milligram over the amount in the control has, however, no significance, for such conditions never occur in the living animal.

In studying the effect of varying amounts of the antibody on the bacteria and their consequent resistance to tryptic digestion, we have at various times observed that bacteria treated with the largest amount of antibody became more resistant to the action of trypsin,

thus in a measure simulating the Neisser-Wechsberg phenomenon. Such an experiment is shown in protocol V.

PROTOCOL V.

Effect of Tryptic Digestion on Organisms Treated with Varying Amounts of Specific and Non-Specific Immune Serum and Complement.

Tube No.	Typhoid bacilli.	Staphylococci.	Typhoid immune serum.	Complement.	Trypsin.	Tryptic digestion.	Increase.
1	1.0 c.c.	.....	Total nitrogen = 1.4 mg., total non-coagulable nitrogen = 0.07 mg. per c.c.				
2	.....	1.0 c.c.	Total nitrogen = 0.715 mg., total non-coagulable nitrogen = 0.16 mg. per c.c.				
3	1.0 c.c.	.....	.....	.....	0.1 c.c.	0.625 mg.	.....
4	.....	1.0 c.c.	.....	.....	0.1 c.c.	0.15 mg.	.....
5	1.0 c.c.	.....	3.0 c.c.	0.3 c.c.	0.1 c.c.	0.54 mg.	−6%
6	1.0 c.c.	.....	1.0 c.c.	0.1 c.c.	0.1 c.c.	0.625 mg.	.....
7	1.0 c.c.	.....	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.65 mg.	+2%
8	1.0 c.c.	.....	0.01 c.c.	0.1 c.c.	0.1 c.c.	0.55 mg.	−5%
9	.....	1.0 c.c.	3.0 c.c.	0.3 c.c.	0.1 c.c.	0.13 mg.	−3%
10	.....	1.0 c.c.	1.0 c.c.	0.1 c.c.	0.1 c.c.	0.20 mg.	+7%
11	.....	1.0 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.24 mg.	+12%
12	.....	1.0 c.c.	0.01 c.c.	0.1 c.c.	0.1 c.c.	0.16 mg.	+1%

In this experiment an emulsion of fresh typhoid bacilli, total nitrogen 1.4 milligrams, and fresh staphylococci, total nitrogen 0.715 of a milligram, were used, permitted to remain with typhoid immune serum and complement for sixteen hours at 37° C., and then washed and digested with 0.1 of a cubic centimeter of trypsin solution. In the controls the typhoid digestion was 0.625 of a milligram, staphylococci digestion 0.15 of a milligram. It will be noted that with three cubic centimeters of the immune serum there was 6 per cent. less digestion than in the control; with one cubic centimeter an equal amount; with 0.1 of a cubic centimeter, a slight increase; while with 0.01 of a cubic centimeter, there was a drop to an amount less than the control. This action was not specific, for when staphylococci and the typhoid immune serum were used a similar and even more pronounced result was obtained. We are inclined to attribute this result to the adsorption by the bacteria of lipoids from the serum, which, if sufficient, as when larger amounts of serum are permitted to act, will protect the bacteria from

tryptic digestion, and overcome the alteration produced by the immune body which under ordinary circumstances seems to render the bacterial body less resistant to trypsin.

In order to avoid the presence of an excess of antibody we have sensitized bacteria with immune serum. They were then washed, treated with complement, washed again, and tryptic digestion of the sensitized and unsensitized bacteria was permitted.

PROTOCOL VI.

Effect of Sensitization of Typhoid Bacilli on Their Tryptic Digestibility.

Tube No.	Typhoid bacteria.	Sensitized typhoid bacteria.	Complement.	Trypsin.	Non-coagulable nitrogen in supernatant fluid.	Tryptic digestion.
1	1.0 c.c.	Total nitrogen = 0.55 mg., total non-coagulable nitrogen = 0.05 mg. per c.c.				
2	.....	1.0 c.c.	Total nitrogen = 0.575 mg., total non-coagulable nitrogen = 0.00 mg. per c.c.			
3	1.0 c.c.	.....	.....	0.1 c.c.	.....	0.162 mg.
4	.....	1.0 c.c.	.....	0.1 c.c.	.....	0.172 mg.
5	1.0 c.c.	.....	1.0 c.c.	.....	0.31 mg.	.....
6	1.0 c.c.	.....	0.1 c.c.	.....	0.12 mg.	.....
7	.....	1.0 c.c.	1.0 c.c.	.....	0.35 mg.	.....
8	.....	1.0 c.c.	0.1 c.c.	.....	Lost	.....
9	.....	.....	1.0 c.c.	.....	0.305 mg.	.....
10	.....	.....	0.1 c.c.	.....	0.06 mg.	.....
11	1.0 c.c.	.....	1.0 c.c.	0.1 c.c.	.....	0.172 mg.
12	1.0 c.c.	.....	0.1 c.c.	0.1 c.c.	.....	0.15 mg.
13	.....	1.0 c.c.	1.0 c.c.	0.1 c.c.	.....	0.316 mg.
14	.....	1.0 c.c.	0.1 c.c.	0.1 c.c.	.....	0.25 mg.

In protocol VI there was no increase in autolysis of the two series of bacteria. This will be seen from tubes 5 to 8, the corresponding serum controls being tubes 9 and 10. The sensitized bacteria are much more easily digested by trypsin (tubes 13 and 14) than the unsensitized bacteria (tubes 11 and 12).

DISCUSSION.

In view of the established fact that the serum of practically all animals contains a markedly active antiferment against trypsin, leucoprotease, and the autolytic ferments,—a non-specific substance which in a neutral or slightly alkaline reaction inhibits protease

action of any kind and is essential to life by preventing the splitting of native proteins in the serum to toxic products,—we can hardly expect a result other than that noted in the above experiments; *i. e.*, the fact that bacteriolysis is not associated with a proteolysis. Jobling and Strouse (7) have called attention to the fact that the mere solution of bacteria should not be confused with proteolysis; the former might be due to purely physical factors, as when organisms are kept under lipoid solvents as preservatives. This distinction should be made with equal emphasis for the immunity reactions.

If bacteriolysis is not associated with proteolysis we must find some other explanation. This, we believe, should be sought in the relation of the lipoids of the bacteria and the lipolytic effect of the serum (Jobling and Bull (8)). That a physical change of some sort is induced in corpuscles treated with immune serum and complement has been shown by Eisner and Friedemann (9), and is indicated by the fact that bacterial organisms are rendered more digestible for trypsin. This might be due to (a) an alteration in the lipoidal surface membranes, rendering the organism more permeable for trypsin; (b) an oxidation, rendering the antiferments of the bacteria less active; or (c) the death of the organism (expressing, of course, a physical change) with resulting alteration of the balance of the bacterial protein and lipoids and consequent loss of antitryptic activity.

Our results might be considered to contradict directly the results obtained by several workers with the Abderhalden dialysis method, who have observed an increase in protein split products when bacteria were allowed to digest with immune serum (Voelkel (10)). These workers have ignored the presence of an excess of antiferment in the blood, assuming that proteolysis might take place under normal conditions with resulting splitting of bacteria. Inasmuch as Plaut (11) has recently shown that such absorbing substances as kaolin, starch, etc., give an equally positive reaction, and since de Waele (12) has just demonstrated the same fact by the simple means of precipitating the globulins of the serum, there can be no question that the idea of a specific protease action is unfounded. This view has also been taken by Kirschbaum and Köhler (13), who have been unable to obtain results by means of the dialysis method which

could in any way be interpreted as indicating a specific protease action upon bacteria. This, of course, does not hold true for the peptases, there being no antiferments in the serum against these ferments. Reasoning from a different line of experiments, based on the demonstration of the lipoidal nature of the antiferments (14), we have shown that serum from which the antiferment has been removed will autolyze (15) and that the antiferment can be removed by adsorption (16), after which there is, of course, no further impediment to either protease or peptase action. It is, therefore, more than probable that the dialyzable split products obtained by various workers from the bacterial digestion by immune serum represent digestion products brought about by an alteration in the balance of the ferment and antiferment when the latter is adsorbed by the bacteria. It is more easy to conceive that the soluble serum proteins are split when their antiferment is adsorbed than that the bacterial protein, protected by its lipoids and membranes, should be attacked.

CONCLUSIONS.

1. There is no demonstrable increase in non-coagulable nitrogen during bacteriolysis.

2. Bacteria treated with immune serum and complement are so altered that they are more readily digested by trypsin.

3. Bacteria treated with complement alone become more resistant to proteolysis.

4. Bacteria treated with an excess of immune serum and complement become more resistant to proteolysis.

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STUDIES ON THE CHEMISTRY OF SEROUS EFFUSIONS.*

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The formation of serous effusions in different diseases and particularly in nephritis has been the subject of much study and discussion. Although the results of analyses of serous fluids recorded in the literature show striking variations in the composition of such fluids in different pathological conditions, they throw but little light on the chemical factors concerned in their formation. This failure to account for the differences in the chemical composition of such fluids is due to two causes: (1) a lack of definite knowledge of the processes by which serous fluids of different chemical composition are formed normally in different parts of the body, and (2) the fact that there are relatively few studies on the subject in which simultaneous examinations of blood serum and serous fluids were made.

Recent studies by the author on the chemical composition of blood serum show that certain changes occur in different diseases.[1] Particularly striking is the change from normal in the composition of the protein colloids and other constituents which takes place in parenchymatous nephritis, in which effusions are of common occurrence, as contrasted with other forms of renal disease, in which effusions do not occur.[2]

Without attempting to enter into a discussion at the present time of the relation of the chemical changes in the blood serum to the nature and occurrence of effusions in disease, I wish to put on record the chemical findings of forty-three puncture fluids, some of which were made simultaneously with analysis of blood serum.

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[1] Epstein, A. A., *Jour. Exper. Med.*, 1912, xvi, 719.

[2] Epstein, *idem*, 1913, xvii, 444.

The analyses of the effusions were carried out along lines similar to those followed in the study of the blood sera.[3] The substances determined were the proteins, chlorides, and incoagulable and non-protein nitrogen. Ash and total solids were also estimated in several of the fluids. A number of the fluids which were opalescent and of a chyliform appearance were analyzed for fats and other ether-soluble substances. These, however, will not be reported in the present study.

The fluids subjected to analysis may be grouped according to the following scheme which is based on clinical data.

1. Cutaneous effusions: (*a*) nephritic and (*b*) cardionephritic.
2. Pleural effusions: (*a*) inflammatory and (*b*) non-inflammatory including (1) nephritic, (2) cardiac, (3) cardionephritic, and (4) new growth.
3. Abdominal effusions: (*a*) inflammatory, tuberculous, and (*b*) non-inflammatory, including (1) new growth, (2) cirrhosis of the liver, (3) cardiac, and (4) cardionephritic.
4. Hydrocele fluid.

CUTANEOUS EFFUSIONS.

TABLE I.

Cutaneous Effusions.

Case No.	Total protein.	Incoagulable nitrogen.	Total globulin.	Euglobulin.	Pseudoglobulin.	Albumin.	Chlorides.	Total solids.	Ash.	Ratio of globulin to albumin.	Per cent. of globulin in protein.
	Gm. per 100 c.c. of fluid.										
	Nephritis.										
4	0.098	0.004	0.080	0.048	0.032	0.018	0.406	1.230	0.980	1:0.24	81
44	0.171	0.162	0.104	0.091	0.095	.....	0.397	.....	.....	1:0.60	56.1
29	0.145	0.063	0.079	0.024	0.055	0.066	0.433	1.357	0.870	1:0.84	54
	Cardionephritis.										
14	0.462	0.035	0.155	0.032	0.123	0.307	0.412	1.515	0.950	1:2.5	28.5
70	0.119	0.035	0.043	0.018	0.025	0.076	0.420	.....	.....	.....	30
201	0.100	0.053	0.018	.....	.....	0.082	0.400	.....	.....	.....	18

The six cutaneous fluids (table I), of which three are of nephritic and three of cardionephritic origin, have certain features in com-

[3] Epstein, *Jour. Exper. Med.*, 1912, xvi, 719.

mon and differ in certain others. The protein content of all these fluids is very low, ranging in the nephritic group from 0.098 of a gram to 0.171 of a gram per 100 cubic centimeters, and in the other group from 0.100 of a gram to 0.462 of a gram per 100 cubic centimeters of fluid. The two classes of fluids differ in the percentage relations of albumin and globulins. Of the three nephritic fluids the globulins constitute 81 per cent., 56.1 per cent., and 54 per cent., respectively, of the total protein. The proportion of globulins in the second group of fluids is much lower; namely, 28.5 per cent., 30 per cent., and 18 per cent., respectively.

Although the amount of protein in these fluids is far below that of blood sera, the percentage of globulin in each group of cutaneous fluids is similar to that of the corresponding group of blood sera. Fluids 4 and 201 are from cases whose blood serum was examined at the same time. Table II shows the values obtained in the analysis of the corresponding serum and edema fluids.

TABLE II.

Case 4. Nephritis.

		Edema fluid.	Blood serum.
Gm. per 100 c.c. of fluid	Total protein	0.098	5.125
	Incoagulable nitrogen	0.040	0.280
	Total globulin	0.080	4.325
	Albumin	0.018	0.800
	Chlorides	0.406	0.412
	Total solids	1.230	.....
	Ash	0.980	.....
	Per cent. of globulin	81.0	83.0
	Case 201. Cardionephritis.		
Gm. per 100 c.c. of fluid	Total protein	0.100	3.330
	Incoagulable nitrogen	0.053	0.154
	Chlorides	0.400	0.435
	Globulin	0.018	2.381
	Albumin	0.082	0.949
	Per cent. of globulin	18.0	71.5

In both cases the quantity of protein in the effusions is very small as compared with that of the blood serum. In the nephritic effusion the percentile relation of the protein fractions corresponds to that of the blood serum. In the cardionephritic case this parallelism does not exist.

The chloride content of these fluids is uniformly high when compared with that of normal blood sera, but much lower than the content in the blood sera of corresponding cases (compare fluids 4 and 201 and their corresponding blood sera). The total solids are low in all the fluids analyzed. The ash, on the other hand, is practically the same as that found in normal blood sera. The incoagulable and non-protein nitrogen in all but one fluid (44) is very low when compared with the blood sera belonging to these cases. In this respect the incoagulable nitrogen is like the chlorides, except that the difference in the quantity of incoagulable nitrogen in the effusions and in the blood sera is much greater than that exhibited by the chlorides.

A comparison of the results obtained in the analyses of the subcutaneous fluids with those of other effusions shows that their composition is unique. They are of a pale watery appearance like lymph, containing very little protein (less than 0.5 per cent.) and a large amount of chlorides. The incoagulable nitrogen is relatively low, certainly far below that found in the blood sera of the cases examined.

PLEURAL FLUIDS.

Most of the pleural fluids analyzed (table III) are of inflammatory origin. Fluids 20, 21, 54, 60, 61, 80, and 204 are from cases of pleurisy of unknown etiology. Fluids 52, 53, and 206 are from cases of probable tuberculosis, and fluid 30 is from a case of empyema. The remaining effusions are presumably of non-inflammatory origin; two (205 and 56) are from cases of nephritis; of the others, 63 is from a case of intrathoracic lymph sarcoma, and 202 and 203 are also due to neoplasms of undetermined nature.

All of the above fluids are rich in protein, the amounts ranging from 1.250 grams to 6.250 grams per 100 cubic centimeters. The inflammatory fluids of unknown etiology (20, 21, 54, 60, 61, 80, and 204) show a moderate elevation of the percentage of globulin as compared with the content of globulin in normal blood serum. The incoagulable and non-protein nitrogen found in these fluids present values which are for the most part somewhat above the standard figure for blood serum. These values vary from 0.049 to 0.080 of a gram per 100 cubic centimeters of fluid. Likewise the chloride content of these fluids is higher than that of normal blood serum.

TABLE III.

Pleural Effusions.

Case No.	Total protein.	Incoagulable nitrogen.	Total globulin.	Euglobulin.	Pseudoglobulin.	Albumin.	Chlorides.	Ash.	Ratio of globulin to albumin.	Per cent. of globulin in protein.
	Gm. per 100 c.c. of fluid.									
Inflammatory. Unknown etiology.										
20	4.400	0.046	1.780	0.470	1.310	2.620	0.362	0.650	1:1.5	40
21	3.969	0.080	1.882	0.392	1.490	2.087	0.355	0.650	1:1.1	47.6
60	3.831	0.050	1.437	0.650	0.787	2.394	0.404	.....	1:1.6	39.3
61	3.412	0.045	2.537	0.569	1.968	0.875	0.412	.....	1:0.34	74.3
54	4.781	0.067	2.056	0.881	1.175	2.715	0.397	.....	1:1.3	43
80	5.487	0.049	2.625	0.822	1.803	2.862	0.412	.....	1:1.1	47.6
204	6.775	0.022	2.187	4.588	.....	.....	0.391	.....	1:2.14	32
Inflammatory. Tuberculosis.										
52	4.275	0.022	2.387	1.181	1.206	1.888	0.372	.....	1:0.8	56
53	6.250	0.056	2.969	0.812	2.157	3.291	0.376	.....	1:1.1	47.6
206	5.231	0.039	3.506	.....	.....	1.725	0.405	.....	1:0.48	67
Inflammatory. Empyema.										
30	5.019	0.086	1.027	0.625	0.402	3.992	0.372	.....	1:3.8	20.8
Non-Inflammatory. Nephritis.										
56	3.581	0.060	1.469	0.631	0.838	2.112	0.426	.....	1:1.4	41
205	1.250	0.164	0.875	.....	.....	0.375	0.426	.....	1:0.3	70
Non-Inflammatory. Neoplasms.										
63	4.250	0.053	1.776	0.490	1.280	2.484	0.390	.....	1:1.4	41.8
202	5.023	0.038	.....	.....	.....	.....	0.440	.....	.....	.....
203	4.950	0.048	.....	.....	.....	.....	0.433	.....	.....	.....

Four fluids in this series are of special interest (20, 21, 60, and 61). They represent fluids which were obtained from two patients upon whom the paracentesis was repeated. Fluids 20 and 21 were removed from the chest of a patient at an interval of three days. Notwithstanding the short interval the two fluids show considerable differences in composition. The fluid from the second paracentesis (21) contains less protein than the first,—3.969 grams as compared with 4.400 grams per 100 cubic centimeters; but the ratio of globulin to albumin is higher by 7.6 per cent. than that present in the fluid from the first paracentesis. The incoagulable nitrogen in the second fluid is almost double that of the first. The chloride content, on the other hand, is slightly lower. The ash content is the same in

both fluids, and if we compute the chlorides in these fluids as sodium chloride, then we obtain values of 0.613 and 0.592 of a gram per 100 cubic centimeters, respectively, which leaves a balance of 0.037 of a gram and 0.058 of a gram per 100 cubic centimeters of fluid for the other salts in the ash.

The second set of effusions show different changes in composition from those observed in the fluids just described. In this instance the second paracentesis was performed twelve days after the first. The fluid from the second paracentesis (61) has a lower protein content than the first (60), *i. e.*, 3.412 grams as compared with 3.831 grams per 100 cubic centimeters. The incoagulable nitrogen falls slightly, whereas the chloride content rises considerably. The most striking change occurs in the percentage of globulin; it rises from 39.3 per cent. to 74.3 per cent. In both sets of analyses it is found that, whereas the percentage of total globulin rises in the second fluid, the percentage of euglobulin to pseudoglobulin falls. This is especially marked in the second set of fluids (60 and 61). It is likely that the cause for the change in the quantitative relations of the globulin rests on a difference in the rate of secretion or resorption of the albumin and globulin fractions of the serum. This, however, will be considered at another time.

The three fluids from the tuberculous cases are in most respects like the other effusions of inflammatory origin. They possess a high protein content. One of these (53) contains an unusually large amount of protein, practically as much as blood serum. It may be added that in this particular fluid a very unusual amount of fibrin was found.

Fluid 30 is from a case of empyema, resulting from a pneumonic infection. This fluid possesses a high protein content, a large amount of incoagulable nitrogen, a small amount of chlorides, and a very low percentage of globulin (20.8 per cent.). In this latter fact we find a contradiction to the usual statement and belief that the serum of purulent fluids is rich in globulins, because of the presence of disintegrating pus cells in the fluid.

In its general character the fluid from a case of intrathoracic lymphosarcoma resembles the fluids that are of inflammatory origin. It does not present any distinguishing features.

The last fluids in this series are from cases of chronic nephritis. In their protein content these are the lowest in the series. The incoagulable nitrogen in one of these (56) is only moderately elevated, whereas the chlorides are rather high. In the second fluid both groups of substances show very high values.

The chemical relation of these fluids to the blood sera of the corresponding cases can be seen in table IV.

TABLE IV.

Case 56.

		Pleural fluid.	Blood serum.
Gm. per 100 c.c. of fluid	Total protein	3.581	7.525
	Incoagulable nitrogen	0.060	0.120
	Total globulin	1.469	2.537
	Albumin	2.112	4.988
	Chlorides	0.426	0.480
	Per cent. of globulin	41.7	34.0
	Case 205.		
Gm. per 100 c.c. of fluid	Total protein	1.250	7.087
	Incoagulable nitrogen	0.164	0.187
	Globulin	0.875	4.775
	Albumin	0.375	2.312
	Chlorides	0.426	0.412
	Per cent. of globulin	70.0	67.0

As table IV shows, striking differences exist between the composition of the effusion fluids, and that of the corresponding blood sera. In one instance (56) the pleural fluid contains less than half of the protein found in the blood serum. In the other case (205) there is even less protein, constituting less than 18 per cent. of that in the blood serum. The incoagulable nitrogen in the first of these fluids is half of that of the corresponding blood serum, in the second it constitutes almost 90 per cent. The chloride in one fluid (56) is lower than the value found in its related blood serum. In the second fluid the chloride content exceeds that of the blood serum.

The percentage of globulin in each fluid corresponds to that of the blood serum from the same case, but in both it is higher than that of the blood serum.

The differences observed in the composition of the pleural fluids and that of the blood sera from the same cases are not easy to

explain. The difference in the amounts of incoagulable nitrogen and chlorides indicates two points: first, that the diffusion of the organic bodies constituting the incoagulable nitrogen is of a different order from that of the inorganic salts; and second, that the difference in the content of these two groups of substances in the pleural fluid and the blood cannot be accounted for by the accepted rules of diffusion and osmosis alone.

ABDOMINAL FLUIDS.

Of the abdominal effusions twenty fluids were examined (table V). Of these, one is of tuberculous origin, two are due to neoplasms of abdominal viscera, nine are due to cardiac insufficiency, four are produced by cirrhosis of the liver, and four occurred in nephritis. One of the latter was from a case of pure nephritis and the other three were from nephritis complicated by disease of the heart.

The character of these fluids is like that of the pleural fluids of the same origin. The two carcinomatous fluids contain moderate amounts of protein, of which half or more is globulin. The incoagulable nitrogen in these two fluids is comparable with the incoagulable nitrogen content of normal blood serum. The chloride content presents no unusual values. In the tuberculous fluid the protein content is moderate in amount, but very much less than that found in pleural fluids of similar origin. The percentage of globulin is equivalent to that of blood serum. The chloride content is high. The other ingredients also closely approximate the values usually obtained in the examination of the blood.

The abdominal fluids of cardiac origin appear to stand in etiologic relationship to static disturbances. The composition of these fluids, with one or two exceptions, is more or less uniform. Their protein content varies between 1.567 and 4.712 grams per 100 cubic centimeters. In seven of the fluids the protein ranges above 3 grams. In nearly all of the latter the globulin content is the same as that found in the serum of normal blood. The same is true of the incoagulable nitrogen. With but two exceptions (7 and 69), the chloride is considerably elevated, ranging from 0.362 of a gram to

0.454 of a gram per 100 cubic centimeters. In one instance (7) the ash was also determined, and was found to be equal to that of normal blood serum.

TABLE V.

Abdominal Effusions.

Case No.	Total protein.	Incoagulable nitrogen.	Total globulin.	Euglobulin.	Pseudoglobulin.	Albumin.	Chlorides.	Total solids.	Ash.	Ratio of globulin to albumin.	Per cent. of globulin in protein.
	Gm. per 100 c.c. of fluid.										
Tuberculous and New Growths.											
105	1.725	0.045	0.281	.....	.....	1.444	0.398	.....	.....	1:5.1	16.3
209	3.725	0.040	1.838	.....	.....	1.887	0.385	.....	.....	1:1.+	49
208	3.681	0.043	1.462	.....	.....	2.219	0.418	.....	.....	1:1.5	39.7
Cardiac.											
7	2.080	0.037	0.882	0.189	0.693	1.198	0.362	3.400	0.958	1:1.36	42.3
28	1.567	0.049	0.692	0.275	0.417	0.975	0.454	.....	.....	1:0.6	62
40	3.675	0.084	1.919	0.625	1.294	1.756	0.412	.....	.....	1:0.9	52.2
69	4.694	0.055	1.787	0.750	1.037	2.907	0.376	.....	.....	1:1.6	38
81	4.712	0.055	1.656	0.456	1.200	3.056	0.404	.....	.....	1:1.9	34
32	3.230	0.053	1.062	0.372	0.690	2.168	0.412	.....	.....	1:2	33.3
66	3.702	0.076	1.462	0.500	0.962	2.240	0.418	.....	.....	1:1.5	39.9
68	3.123	0.077	1.331	0.463	0.868	1.792	0.404	.....	.....	1:1.3	42.6
207	3.381	0.040	.....	.....	.....	.....	0.440	.....	.....	.....	.....
Cirrhosis of the liver.											
15	3.332	0.090	1.625	0.525	1.100	1.707	0.428	4.815	0.950	1:1.05	49
26	3.017	0.051	1.012	0.205	0.707	2.005	0.390	.....	.....	1:2.0	33.3
67	0.521	0.039	0.325	0.125	0.200	0.193	0.326	.....	.....	1:0.6	62.3
79	0.686	0.038	0.400	0.131	0.769	0.256	0.497	.....	.....	1:0.6	62.3
Nephritis.											
1a	0.285	0.035	0.285	.....	.....	.....	0.420	1.040	0.875	.....	100
Cardionephritis.											
32	3.230	0.053	1.062	0.372	0.690	2.168	0.412	.....	.....	1:2	33.3
66	3.702	0.076	1.462	0.500	0.962	2.240	0.418	.....	.....	1:1.5	39.9
68	3.123	0.077	1.331	0.463	0.868	1.792	0.404	.....	.....	1:1.3	42.6

In one case (table VI) both the blood serum and the ascitic fluid were examined. The protein content of the ascitic fluid is less than half of that of the blood serum, but the percentage of globulin is nearly double that of the other. In other respects the two fluids are alike.

Of the fluids arising in cases of cirrhosis of the liver, two (15 and 26) resemble the abdominal fluids of cardiac origin; the others differ considerably. The first two contain over 3 grams of protein per 100 cubic centimeters of fluid, and the percentage of globulin is the same as that found in the fluids of cardiac origin. The same

TABLE VI.

Case 207.

		Ascitic fluid.	Blood serum.
Gm. per 100 c.c. of fluid	Total protein.....	3.381	7.575
	Incoagulable nitrogen....	0.040	0.040
	Globulin.....	1.481	1.956
	Albumin.....	1.900	5.619
	Chlorides.....	0.440	0.433
	Per cent. of globulin.....	43.0	26.0

is true of the incoagulable nitrogen and chloride contents. In the other two fluids (67 and 79) the protein content is rather low,—slightly over 0.5 of a gram per 100 cubic centimeters,—but the globulin fraction is very high in both, being 62.3 per cent. The incoagulable nitrogen in these two effusions is low, or comparable to that of normal blood serum. The chloride content in one fluid (67) is very low, 0.326 of a gram per 100 cubic centimeters, in the other exceedingly high, 0.497 of a gram per 100 cubic centimeters. It would have been of interest to compare the quantities of the different ingredients with those of the blood serum of the corresponding cases, but these were not available.

Four abdominal fluids of renal origin were examined. Of these only one is from a case of pure nephritis. The others are from cases of nephritis complicated by heart disease. The latter resemble the composition of the fluids of cardiac origin described above. The protein content is over 3 grams to 100 cubic centimeters. The incoagulable nitrogen is slightly elevated; the chloride content is high, but unlike the other fluids. The globulin percentage is low, averaging 38.6 per cent. In this the fluids resemble normal blood serum. The fluid of purely nephritic origin (1a) contains a small amount of protein (0.285 of a gram to 100 cubic centimeters), but all of it is globulin. In this respect this fluid is different from all the other fluids examined excepting the subcutaneous effusions

of nephritic origin. The incoagulable nitrogen is low, but the chloride content is very high, 0.420 of a gram per 100 cubic centimeters. The value obtained in the analysis of the ash is low and considerably under that of normal blood serum. If we deduct from the quantity of ash the salt equivalent of the chlorides in it, there remains but a small balance for other salts.

The abdominal fluid just described gains in interest when the results are compared with values obtained in the analysis of the blood sera from nephritic cases.[4]

HYDROCELE FLUID.

One hydrocele fluid was also examined; the results are shown in table VII. This fluid is rich in protein, but its globulin content is

TABLE VII.

Hydrocele Fluid.

Case No.	Total protein.	Incoagulable nitrogen.	Total globulin	Euglobulin.	Pseudoglobulin.	Albumin.	Chlorides.	Total solids.	Ash.	Ratio of globulin to albumin.	Per cent. of globulin in protein.
	Gm. per 100 c.c. of fluid.										
17	5.332	0.061	1.100	0.270	0.830	4.232	0.412	6.960	0.945	1:3.8	20.7

low. The chlorides are rather high, as is the ash. The other ingredients present nothing striking.

SUMMARY.

A comparison of the results obtained in the analysis of the different effusions shows that they vary, (1) according to location, and (2) according to the disease in which they are produced. Thus the subcutaneous effusions are totally different in their composition from the abdominal or pleuritic fluids; and again the abdominal fluid of nephritic origin is different from those of cardiac or other origin.

The cutaneous effusions are characterized by a very low protein content and a small amount of incoagulable nitrogen. Of the protein present in these fluids, the globulin constitutes the greater

[4] Epstein, *Jour. Exper. Med.*, 1912, xvi, 719.

portion. In the mixed cardionephritic fluids the ratio of globulin to albumin is lower than that in normal blood serum; but when compared with the blood sera of cardionephritic cases there appears to be a certain parallelism. The chloride content of these fluids is considerably above that found in normal blood serum, but is comparable with those of corresponding cases.

The effusions occurring in serous cavities differ from the cutaneous ones by their higher protein content. The highest values are attained in the pleural fluids, in which the protein present is almost the same as that in normal blood serum. The incoagulable nitrogen in these fluids is uniformly low. The chlorides vary in amount according to the nature of the case. In the effusions of inflammatory origin the values are lower than those found in the blood. In the pleural fluids of other than inflammatory origin the chloride content is either the same or higher than that of blood serum. The globulin-albumin ratio in all of these fluids except one[5] (table III, fluid 61) is higher than that of normal blood serum. Otherwise the highest value is presented by a pleural fluid from a case of nephritis (table III, fluid 205) and the lowest by an empyema fluid (table III, fluid 30). The latter case is of especial interest because of the theory, first propounded by Schmidt, that the leucocytes in the purulent fluid are largely responsible for high globulin content.

The abdominal fluids in general are less rich in protein than the pleural effusions. Those of cardiac origin give the highest protein values, but the globulin-albumin ratios are lower than in the pleural fluids or in the blood sera of corresponding cases. Only in two fluids does the globulin reach a percentage of 62 and 52.2. In both these cases the chloride content is also high. The incoagulable nitrogen in all of them is rather low. In one fluid of purely nephritic origin the protein content is low and all of it is globulin. This is suggestive, when compared with the values obtained in the analysis of the blood sera from nephritic cases. The results obtained in the analysis of abdominal fluids from cardionephritic cases approach very closely those obtained in purely cardiac cases.

[5] This fluid was obtained at a second tapping. The fluid obtained at the first paracentesis is shown in table III, fluid 60.

PNEUMOCOCCUS HEMOTOXIN.*

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It has been generally held that pneumococci or their products do not possess the power of producing lysis of red blood cells. On the other hand, it has long been known that the culture filtrates of certain bacteria, such as staphylococcus, certain races of streptococcus, and, above all, *Bacillus tetani,* possess this property to a marked degree. The properties of the hemolytic toxin produced by the last named organisms have been thoroughly studied by Madsen and others, and through this study many of the facts concerning hemolytic toxins have been discovered. Since such hemolytic toxins may be filtered, and since they may act as antigens, they may be considered true toxins in the Ehrlich sense. Old cultures of other bacteria, such as *Bacillus pyocyaneus* and *Bacillus anthracis,* may also be hemolytic, but the production of true hemolytic toxins by these organisms is considered doubtful. Indeed, very old cultures of practically all bacteria may produce hemolysis, but it is possible that this lytic effect, in certain of the cases at least, is due directly to changes in reaction of the old culture medium.

The property of certain races of streptococci of producing hemolysis has been considered by Schottmüller and others to be of great value in differentiating between the different varieties, and also between pneumococcus and the virulent streptococci, the so called *Streptococcus hemolyticus.* The usual method of determining whether bacteria possess this property is to grow them on agar plates containing blood, when in the case of hemolytic bacteria, such as *Streptococcus hemolyticus,* the colonies become surrounded by a transparent zone contrasting with the opacity of the rest of the medium, while in the case of non-hemolytic bacteria, such as pneumococcus, no such clear zones are seen.

* Received for publication, August 1, 1914.

While the power to produce hemolysis in culture medium is not possessed by pneumococci, or, if so, to a very slight degree, observations which we have made indicate that the bodies of pneumococci contain a substance or substances, which when set free are actively hemolytic, and that the serum of animals immunized to the bacteria or to the bacterial substance has increased power of neutralizing this lytic poison. These lytic substances differ from the so called bacterial hemotoxins in that they are contained within the bacterial cells and are only set free on the dissolution of the latter, but they may, nevertheless, have as great a pathological significance.

The first observations were made when studying the properties of a poison produced by dissolving washed pneumococci in dilute solutions of bile or sodium cholate.[1] The solution so obtained, which produces acute death in guinea pigs on intravenous injection, was also found to be hemolytic when added to an emulsion of red blood corpuscles in salt solution. At first this hemolytic property was thought to be due to the sodium cholate contained in the solution, but careful titration of the hemolytic power of the toxin showed that it was much greater than could be accounted for by the contained sodium cholate. In certain experiments, three hundred times as much sodium cholate in salt solution was required to produce hemolysis of 0.5 c.c. of an emulsion of sheep corpuscles as was contained in a minimal lytic dose of the toxin. It is not likely that the mere presence of bacterial substance increases the activity of cholate solutions, since the addition of protein to a cholate solution lessens its activity, and even peptone was found to have no intensifying action, but a slightly inhibiting action instead.

Later experiments have shown conclusively that cholate plays no important part in the reaction, since the lytic substances are present in extracts of pneumococci, obtained by allowing pneumococci to undergo autolysis in salt solution and also in extracts prepared by freezing and grinding the bacteria, in both cases without adding any cholate whatsoever.

PREPARATION OF THE TOXIN.

In the study of the lytic effect of substances obtained from the bodies of pneumococci, the extracts have been prepared in one of the following ways:

(1) Pneumococci are grown for twenty hours in broth, removed from the broth by centrifugalization, and washed once in 0.85 per

[1] Cole, R., *Jour. Exper. Med.*, 1912, xvi, 644.

cent. salt solution. A test of the hemolytic power of toxins made from cultures grown for various lengths of time has shown that the toxins made from cultures twenty to twenty-four hours old are the most active. Toxins made from forty-eight-hour cultures possess little hemolytic power, while those made from seventy-two-hour cultures have no lytic power whatever. This difference is probably associated with the lysis of the bacteria which goes on in old cultures. The washed bacterial sediment is taken up in a small amount of salt solution, usually ten cubic centimeters for the bacteria from one liter of broth culture, and an amount of a 2 per cent. sodium cholate solution barely sufficient to cause solution of the bacteria is added. Usually one cubic centimeter is sufficient. After lysis has occurred, the solution is diluted with salt solution. If the bacteria were obtained from one liter of culture, the solution is usually made up to one hundred cubic centimeters. Different races of bacteria differ in the readiness with which they dissolve in cholate solution. Moreover, different solutions of bacterial bodies obtained in this way differ in their power to produce death in guinea pigs and also to produce hemolysis. Toxins, as above described, however, usually produce acute death in guinea pigs in doses of three to four cubic centimeters, and hemolysis of 0.5 of a cubic centimeter of sheep corpuscles in doses of 0.02 of a cubic centimeter or less, the whole mixture of toxin and corpuscles being made up to 2.5 cubic centimeters with salt solution.

(2) Pneumococci are grown in broth, washed, frozen, and ground, and the powder is dissolved in salt solution. A diluted toxin so prepared, which kills a guinea pig acutely in doses of three to four cubic centimeters, also usually produces hemolysis of sheep corpuscles, with the technique above described, in doses of 0.02 of a cubic centimeter or less.

Toxins prepared in the above ways are identical in their reactions so far as studied, and in the following pages no mention will be made of the method of preparation in each individual experiment.

A few experiments have also been conducted with extracts prepared by allowing pneumococci to undergo autolysis in salt solution. A series of tubes were prepared, all containing equal quantities of washed pneumococci and equal quantities of salt solution. These

were kept at 37° C., and from time to time a tube was removed and the hemolytic power of the fluid tested. It was found that the hemolytic power of the emulsion rapidly increased and between six and eight hours was at its maximum. There was a very slight fall in hemolytic power up to eighteen hours, but at twenty-four hours it had markedly diminished and was entirely absent after forty-eight hours.

PROPERTIES OF THE TOXIN.

Toxins prepared by solution of pneumococci are lytic for rabbit, sheep, guinea pig, and human red blood corpuscles. Other corpuscles have not been tested. The hemolytic power is greatest for guinea pig corpuscles, less for sheep and human corpuscles, and least for rabbit corpuscles, but the differences are not striking. The rate of hemolysis depends upon the concentration of the toxin. If the concentration be sufficiently great, complete hemolysis may occur within five to ten minutes at 37° C.

Active toxins have been obtained from pneumococci belonging to all of the four immunological groups.[2]

An attempt has been made to discover whether or not any relationship exists between the virulence of organisms employed and the hemolytic power of the extract, and a larger number of races have been studied with this point in mind. Rosenow[3] has stated that the more virulent races of pneumococci autolyze most readily. This is generally true, but there are many exceptions. Also the more virulent races are more soluble in bile, though to this also there are apparent exceptions. When a series of cultures of pneumococci are tested, these two properties do not bear constant positions with reference to the virulence of the organisms. It may also be stated from our study that the most active hemolytic toxins are usually obtained from those races of pneumococci that have been most lately cultivated from the animal body and are most virulent. But here again there are sufficient exceptions to throw some doubt on the validity of the generalization. The attempt to increase the hemolytic power of the toxin produced from a given race by repeated passage through animals, testing the toxin production from time to time, does not

[2] Dochez, A. R., and Gillespie, L. J., *Jour. Am. Med. Assn.*, 1913, lxi, 727.
[3] Rosenow, E. C., *Jour. Infect. Dis.*, 1912, x, 113.

yield results that are uniform and consistent. With a given race the hemolytic power of the toxin varies markedly from time to time, even though in each test the toxin is prepared in exactly the same way. This variation probably depends somewhat on the luxuriance of the growth, and this on many factors,—the exact composition of the medium, the exact temperature of the thermostat, etc. The amount of autolysis that has gone on in the culture before centrifugalization is also of importance. It is therefore impossible to predict with any given race and culture exactly the strength of the toxin that will be obtained. The conditions are quite different from those obtaining in the production of diphtheria toxin with different races of bacilli. Here races vary markedly in their power to produce toxin, but this property seems to be fixed in certain races, and no matter how long they are grown outside the body or under what unusual conditions, the power of producing toxin in large amounts is preserved.

As regards the relationship between the hemolytic power and the toxic power as tested by intravenous injection into guinea pigs, there is a much more constant relationship. Even here the parallelism is not exact, but actively hemolytic solutions have always been found to be toxic and, with a few exceptions, the reverse is true. During the past two years toxins from a large number of cultures have been tested, and from the results obtained the above conclusions are derived.

The same manipulations which affect the hemolytic power of the toxin also affect the toxic power. It is impossible to draw an absolute conclusion from this that the same substance which produces hemolysis is the cause of the fatal effect in guinea pigs, though this is strongly suggested. On the other hand, symptoms and pathological changes in the guinea pigs do not seem to indicate that the animals die from the effects of hemolysis alone. In these animals hematuria frequently occurs and focal hemorrhages are seen post mortem, but there are no indications of a wide-spread hemolysis either when death occurs acutely or only after several hours. It is possible that, as in tetanus toxin, the effects may be due to two substances occurring together.

The effect of heat, acids, etc., on the toxin as tested by its power

to produce hemolysis, corresponds with the previously reported effects of these agents on the poison as tested by injection into guinea pigs.[4] Heating to 55° C. for one half hour in our experience always destroys the hemolytic power of the toxin. Heating for one and one half hours at 45° C. usually has no effect, though in one experiment the hemolytic power was diminished after heating one half hour at 45° C. Rosenow[5] has stated that if the autolysate of pneumococci be plunged into boiling water and boiled for ten minutes, then quickly cooled in ice water, the toxicity for guinea pigs frequently remains. As far as the hemolytic effect is concerned, this statement does not hold good, for boiling for ten minutes and then plunging into ice water completely destroys its activity. The hemolytic power is slowly lost when the toxin is kept for some time on ice, though the change does not begin until after eighteen to twenty-four hours.

The hemolytic effect of the solution is markedly diminished or entirely lost after passing through a Berkefeld filter. However, even where controls have shown that the filters entirely prevent the passage of bacteria, the filtrate may still possess some hemolytic power, but it is diminished.

Digestion of the hemolytic toxin with trypsin destroys its activity within forty-five minutes. This is shown by the following experiment.

Fairchild's trypsin solution was employed, each cubic centimeter of which contains 300 units. Even dilute solutions of sodium hydroxide may cause hemolysis, so in making these tests a 5 per cent. solution of sodium carbonate was used to render the mixture alkaline.

The following mixtures were prepared, using toxin prepared from frozen and ground bacteria:

(*a*) 12 c.c. toxin.
3 c.c. 0.85 per cent. sodium chloride solution.

(*b*) 12 c.c. toxin.
1.5 c.c. trypsin solution.
1.5 c.c. 0.5 per cent. solution sodium carbonate.

Two series of tests were made (table I). In series 1 the mixtures were made up cold and the sheep corpuscles were added at once. In series 2 the mixtures were incubated at 37° C. for 45 minutes before the corpuscles were added.

[4] Cole, R., *Jour. Exper. Med.*, 1912, xvi, 644.
[5] Rosenow, E. C., *Jour. Infect. Dis.*, 1912, xi, 235.

TABLE I.[6]

Series 1.

Tube No.	1 c.c. of mixture in dilution.	Sodium chloride solution.	Emulsion of sheep corpuscles.	Results. Readings after 3 hrs. at 37° C.	
				Mixture (*a*).	Mixture (*b*).
1	1 : 2	1 c.c.	0.5 c.c.	+	+
2	1 : 4	1 c.c.	0.5 c.c.	+	+
3	1 : 8	1 c.c.	0.5 c.c.	+	+
4	1 : 16	1 c.c.	0.5 c.c.	+	+
5	1 : 32	1 c.c.	0.5 c.c.	+	+
6	1 : 64	1 c.c.	0.5 c.c.	+	+
7	1 : 128	1 c.c.	0.5 c.c.	±	±
8	1 : 256	1 c.c.	0.5 c.c.	ϕ	o
9	1 : 512	1 c.c.	0.5 c.c.	o	o
Series 2.					
1	1 : 2	1 c.c.	0.5 c.c.	+	o
2	1 : 4	1 c.c.	0.5 c.c.	+	o
3	1 : 8	1 c.c.	0.5 c.c.	+	o
4	1 : 16	1 c.c.	0.5 c.c.	+	o
5	1 : 32	1 c.c.	0.5 c.c.	+	o
6	1 : 64	1 c.c.	0.5 c.c.	+	o
7	1 : 128	1 c.c.	0.5 c.c.	+	o
8	1 : 256	1 c.c.	0.5 c.c.	±	o
9	1 : 512	1 c.c.	0.5 c.c.	±	o

At 37° C. for 45 min.

Controls made with toxin plus sodium carbonate and with sodium carbonate alone showed that the latter had no effect on hemolysis.

The fact that the action of trypsin destroys the hemolytic effect of the solution obviously does not prove that the toxin is of protein nature. It indicates, however, that the toxin is probably closely associated with the protein constituents.

Attempts to extract the toxic substance with ether have so far proved unavailing. The toxin has been extracted with large amounts of ether, the ether evaporated under a fan in the cold, and the oily residue taken up in a small amount of alcohol and made into an emulsion in salt solution. Such an emulsion, however, has not been found hemolytic. There is no evidence, therefore, that the hemolytic effects are due to fatty or lipoidal constituents of the bacterial cells.

The presence of blood serum is known to inhibit the action of certain hemolytic toxins, not only bacterial toxins but others as well. Experiments with normal serum showed that the action of the pneumococcus hemolytic toxin is also inhibited to some extent by the

[6] In the tables + indicates complete hemolysis; ± indicates partial hemolysis; ϕ indicates a trace of hemolysis; o indicates no hemolysis.

presence of normal horse serum, to a somewhat greater extent by normal sheep and normal human serum, and to a still greater extent by normal rabbit serum. This antihemolytic effect is also possessed by dilute solutions of egg albumen. The effect of mucus contained in the sputum of a patient suffering from pneumonia was also tested. The mucus was shaken in salt solution; this mixture was added to the toxin and after one half hour at 37° C., sheep corpuscles were added. It was found that the mucus also had marked inhibiting power. Noguchi[7] has brought evidence to show that the inhibiting effect of normal blood serum for tetanolysin is due, in part at least, to the presence of cholesterin. It was therefore important to determine whether or not the hemolytic effect of the pneumococcus toxin was inhibited by the presence of cholesterin.

A protocol of one of the experiments to determine the effect of cholesterin in inhibiting hemolysis is given below.

Toxin.—June 17, 1914. Toxin was prepared by adding the washed bacteria from 500 c.c. of a 20-hour broth culture of pneumococcus A69 to 2.5 c.c. of salt solution plus 1 c.c. of a 2 per cent. solution of sodium cholate, placing the mixture at 37° C. for 15 minutes and then adding salt solution to 50 c.c.

Cholesterin Emulsion.—Cholesterin crystals were dissolved in a small amount of warm ether, and then sufficient warm sodium chloride solution was added, shaking constantly, to obtain a 1 per cent. emulsion. This was heated over a steam bath for 30 minutes to drive off the ether and filtered.

The experiments (table II) have shown conclusively that exceedingly small amounts of cholesterin are able to inhibit the action of the toxin. It is probable that the inhibiting effect of serum is also due to its cholesterin content. It is unnecessary to discuss the theoretical aspects of this phenomenon here, since the problem has been thoroughly considered by Noguchi and others in connection with the inhibition of tetanus hemolysis by serum. As they have concluded, this inhibition by cholesterin probably indicates that the lipoidal constituent of the red blood cell plays an important part in hemolysis.

It also seemed of importance to determine the effect of lecithin on the hemolytic action of this toxin. Kyes[8] has shown that the presence of lecithin increases the hemolytic action of cobra venom,

[7] Noguchi, H., *Univ. Penn. Med. Bull.*, 1902, xv, 327.

[8] Kyes, P., *Berl. klin. Wchnschr.*, 1902, xxxix, 918.

TABLE II.

Hemolytic Test with Toxin.

Tube No.	Experiments.	Hemolysis.	
		1 hr. at 37° C.	24 hrs. on ice.
1	1 c.c. toxin undiluted + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	+	+
2	1 c.c. toxin diluted (1 : 2) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	±	+
3	1 c.c. toxin diluted (1 : 4) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	+	+
4	1 c.c. toxin diluted (1 : 8) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	+	+
5	1 c.c. toxin diluted (1 : 16) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	+	+
6	1 c.c. toxin diluted (1 : 32) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	+	+
7	1 c.c. toxin diluted (1 : 64) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	+	+
8	1 c.c. toxin diluted (1 : 128) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	±	±

Test of Inhibition with Cholesterin.

1	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 100)	30 min. at 37° C. 0.5 c.c. sheep corpuscles added to each tube.	0	0
2	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 500)		0	0
3	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 1,000)		ø	0
4	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 5,000)		0	0
5	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 10,000)		0	0
6	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 50,000)		ø	±
7	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 100,000)		±	±
8	1 c.c. toxin diluted (1 : 25) + 1 c.c. sodium chloride solution		+	+
1	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 100)	5 min. at 37° C. 0.5 c.c. sheep corpuscles added to each tube.	0	0
2	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 500)		0	0
3	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 1,000)		0	0
4	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 5,000)		0	0
5	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 10,000)		0	ø
6	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 50,000)		ø	±
7	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 100,000)		+	+
8	1 c.c. toxin diluted (1 : 25) + 1 c.c. sodium chloride solution		+	+

the lecithin acting as an activator. He[9] has also been able to form a combination of the active constituent of cobra venom with lecithin. On the other hand, it has been shown that with other hemolytic toxins lecithin may have an inhibiting action.

The following protocols of experiments indicate the effect of lecithin on the pneumococcus hemolytic toxin (table III).

TABLE III.

Tube No.	Experiments of July 2, 1914.		Hemolysis.
1	1 c.c. toxin A69 diluted (1 : 32) + 1 c.c. 1% lecithin emulsion diluted (1 : 10)	30 min. at 37° C. 0.5 c.c. emulsion of sheep corpuscles added to each tube.	ø
2	1 c.c. toxin A69 diluted (1 : 32) + 1 c.c. 1% lecithin emulsion diluted (1 : 50)		ø
3	1 c.c. toxin A69 diluted (1 : 32) + 1 c.c. 1% lecithin emulsion diluted (1 : 100)		ø
4	1 c.c. toxin A69 diluted (1 : 32) + 1 c.c. 1% lecithin emulsion diluted (1 : 500)		+
5	1 c.c. toxin A69 diluted (1 : 32) + 1 c.c. sodium chloride solution		+
6	1 c.c. toxin A69 diluted (1 : 64) + 1 c.c. sodium chloride solution		+
7	1 c.c. toxin A69 diluted (1 : 128) + 1 c.c. sodium chloride solution		+
8	1 c.c. toxin A69 diluted (1 : 256) + 1 c.c. sodium chloride solution		±

The tubes were kept for 1 hr. at 37° C. and 24 hrs. on ice.

The lecithin emulsion was prepared by making a 1 per cent. solution of Merck's lecithin in methyl alcohol, and of this a 10 per cent. emulsion was made in 0.85 per cent. salt solution.

This and other similar experiments have shown that lecithin in low dilutions has slight inhibiting action on the hemolytic effect of the toxin.

To learn whether non-hemolytic doses were rendered hemolytic by the presence of lecithin, experiments like the following were made (table IV).

From this and a number of similar experiments, it is evident that lecithin in no case increases the action of the hemolytic toxin. Except for the slight inhibiting action previously noted, therefore, lecithin has no effect on the hemolytic action of the toxin.

[9] *Kyes, P., Berl. klin. Wchnschr.*, 1903, xl, 956, 982.

TABLE IV.

Tube No.	Experiments of March 31, 1914.	Hemolysis.
1	1 c.c. toxin (1 : 10) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	+
2	1 c.c. toxin (1 : 50) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	+
3	1 c.c. toxin (1 : 100) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	±
4	1 c.c. toxin (1 : 500) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	0
5	1 c.c. toxin (1 : 500) + 1 c.c. lecithin emulsion (1 : 10) + 0.5 c.c. sheep corpuscles	0
6	1 c.c. toxin (1 : 1,000) + 1 c.c. lecithin emulsion (1: 10) + 0.5 c.c. sheep corpuscles	0
7	1 c.c. sodium chloride + 1 c.c. lecithin emulsion (1 : 10) + 0.5 c.c. sheep corpuscles	0

The tubes were kept for 2 hrs. at 37° C.

NATURE OF THE HEMOLYTIC TOXIN.

It is not the purpose of the present paper to discuss the chemical nature of the toxin or its mode of action. Sufficient study of it has not been made to render such a discussion profitable. From the properties already described it is evident that it is closely related to certain other vegetable and animal hemolytic toxins. It is extremely labile, is readily absorbed (as shown by the difficulty with which it passes through a bacterial filter), is destroyed by the action of trypsin, and its action is prevented by the presence of minute amounts of cholesterin and of larger amounts of lecithin.

To the view that it does not exist preformed in the bacterial cell, but is a product arising during self digestion or autolysis, the conclusive objection may be raised that it is present in the solutions prepared by freezing, drying, and grinding the bacteria. During this process there has been no opportunity for autolysis and the conclusion seems justified that the hemolytic substance is contained within the living bacterial cell.

ANTIHEMOLYTIC SERA.

In order to demonstrate that the hemolytic substance under discussion is of the nature of a true toxin, it is necessary to show that it possesses antigenic properties; that is, that its action is inhibited by the serum of animals immunized to it. For purposes of immuniza-

tion rabbits and sheep were employed. These were injected intravenously with increasing doses of the toxin every seven to eight days. The toxins for injection were prepared by dissolving the pneumococci in sodium cholate solution, according to the method previously described, and they were centrifugalized before injection. It is probable that with each injection a few living organisms were also introduced. Since the serum of these animals acquired no agglutinating power for the homologous organisms, however, it is not likely that the acquired properties of the serum were due to the antigenic properties of these few bacteria.

(*a*) *Immune Rabbit Serum.*—The protocol of one experiment is given below (table V).

Rabbit 82–E.—Immunization commenced June 14, 1912. Received 5 doses of toxic extract intravenously during a period of 4 months. The animal received no further injections until June 5, 1913. It then received 7 doses of toxin intravenously at intervals of 6 to 7 days, the last injection being made on July 19. Bled on July 31.

Rabbit 285–A.—Immunization commenced June 12, 1913. Received 7 increasing doses of toxin intravenously, the last one being given on July 19. Animal bled on July 31.

TABLE V.

Sera Tested July 31, 1912. Toxin Prepared from Homologous Organisms.

Experiments.		Hemolysis.			
		Serum 82–E.	Serum 285–A.	Normal rabbit serum.	No serum.
Toxin diluted (1 : 8) + serum diluted (1 : 10)	½ hr. at 37° C.	0	0	±	
Toxin diluted (1 : 8) + serum diluted (1 : 50)		0	0	+	
Toxin diluted (1 : 8) + serum diluted (1 : 100)		0	0	+	
Toxin diluted (1 : 8) + serum diluted (1 : 500)		±	±	+	
Toxin diluted (1 : 8) + serum diluted (1 : 1,000)		±	+	+	
Toxin diluted (1 : 8) + sodium chloride					+

The experiments have been repeated many times with the sera of six rabbits immunized to this toxin. While in no case has the antihemolytic power of the immune serum been greater than 0.002 of a cubic centimeter to two hemolytic units of toxin, in all experiments the antihemolytic power of the immune serum has been considerably

greater than that of normal rabbit serum. Tests of the rabbit sera for agglutinins were made from time to time, but none of the sera acquired the power of agglutinating the homologous organisms.

(*b*) *Immune Sheep Serum.*—Three sheep have been given increasing doses of extracts of pneumococci. Two of these animals were treated with extracts of pneumococci of type I and one received injections of pneumococci of type III, the so called *Pneumococcus mucosus.* The results obtained from the study of the sera of these sheep were identical, and data concerning the serum of but one are given.

Sheep A.—Immunization was commenced May 29, 1913. The toxins for injection were prepared by dissolving pneumococci in sodium cholate solution, as previously described, and the injections were all made intravenously. During the period of about 6½ months the sheep received 18 injections of the toxin. The first injection of the toxin was prepared from the bacteria contained in 12.5 c.c. of a twenty-four-hour bouillon culture. The injections were gradually increased in size. On Dec. 2, 1913, the toxin injected was prepared from the bacteria contained in 1,900 c.c. of a twenty-four-hour bouillon culture. On Dec. 10, an injection was made of toxin prepared from the bacteria contained in 3,000 c.c. of a twenty-four-hour bouillon culture. This injection was apparently too large and probably contained a considerable number of living organisms. Following this injection the animal appeared sick, the temperature was elevated, and the respirations were rapid. After a few days' illness the animal appeared better, but a cough persisted for 2 months with gradual loss of weight and strength, and it died Feb. 10, 1914.

Autopsy.—The pleural and pericardial sacs showed extensive fibrous adhesions. The lungs were edematous. Smears and cultures from the heart's blood showed the presence of pneumococci and a gas-forming anaerobic bacillus morphologically like *Bacillus tetani.* Unfortunately, through an error the cultures were destroyed before the identity of the latter organism could be accurately determined.

TESTS OF SERUM OF SHEEP A.

Agglutination.—The serum was repeatedly tested for agglutination with the homologous organism, the last test being made with serum obtained on December 2, 1913. At no time did the serum possess any agglutinating power.

Protection.—Repeated tests of the protective power of the immune sheep serum for mice were made, employing the technique used in this laboratory for determining protective power.[10] It was found that the serum possessed fairly well marked protective power against pneumococci of type I, and some protective power against

[10] Dochez, A. R., *Jour. Exper. Med.*, 1912, xvi, 665.

pneumococci of other types, except type III, against which no protective power is ever present. The protective power, however, was never so high as that of the serum of horses immunized by injection of living organisms, nor was it so specific.

TABLE VI.

Sheep Serum A, Obtained September 14, 1913. Inactivated. Tested September 15, 1913. Toxin 1.70.

Tube No.	Experiments.		Hemolysis.	
			Normal sheep serum.	Immune sheep serum A.
1	1 c.c. toxin 1.70 + 1 c.c. serum diluted (1 : 10) ...	½ hr. at 37° C. 0.5 c.c. emulsion sheep corpuscles added to each tube.	0	0
2	1 c.c. toxin 1.70 + 1 c.c. serum diluted (1 : 50) ...		ø	0
3	1 c.c. toxin 1.70 + 1 c.c. serum diluted (1 : 100) ..		ø	0
4	1 c.c. toxin 1.70 + 1 c.c. serum diluted (1 : 500) ..		±	0
5	1 c.c. toxin 1.70 + 1 c.c. serum diluted (1 : 1,000).		+	0
6	1 c.c. toxin 1.70 + 1 c.c. serum diluted (1 : 5,000).		+	±
7	1 c.c. toxin 1.70 + 1 c.c. serum diluted (1 : 10,000)		+	+
8	1 c.c. toxin 1.70 + 1 c.c. sodium chloride		+	+

The tubes were kept for 1 hr. at 37° C. and 24 hrs. on ice.

Antihemolytic Power.—(Table VI.) This and many similar experiments indicate that the serum of an animal immunized by the injection of toxin possesses an increased antihemolytic power. This antihemolytic action is not highly specific, however, as regards the type of organisms; a serum produced by the injection of toxin prepared from pneumococci of type I protects almost as well against a toxin prepared from organisms of type II as against one prepared from pneumococci of homologous type.

In immunization in this manner, the possibility cannot be excluded that in addition to the introduction of the toxin living organisms have also been introduced. It was therefore important to know whether or not an immune serum produced by the injection of living organisms would or would not have an effect on inhibiting the action of the hemolytic toxin. For this purpose the sera of horses immunized by the injection of living pneumococci were studied.

(*c*) *Immune Horse Serum.*—The horses from which these sera were obtained were immunized by injecting intravenously repeated and increasing numbers of living pneumococci previously washed

in salt solution. These bacteria were obtained by centrifugalization from bouillon cultures. Usually one or two doses of pneumococci killed by heat were injected before proceeding to the injection of living bacteria. The sera studied were those that have been used in the treatment of patients and were active in protection only against the organisms of the type used in immunization. The sera were actively and specifically agglutinating.

Serum I had such protective power that when 0.2 of a cubic centimeter of the serum was mixed with 0.1 of a cubic centimeter of a twenty-hour bouillon culture of organisms of type I and the mixture was injected into a mouse, the mouse lived; whereas 0.000001 of a cubic centimeter of the culture injected alone killed within twenty-four hours.

Serum II had a little less protective power, in that 0.2 of a cubic centimeter of serum protected against only 0.01 of a cubic centimeter of culture of which 0.000001 of a cubic centimeter injected alone killed within twenty-four hours.

Tests of the antihemolytic power gave results similar to those obtained in an experiment of which the following is the protocol (table VII).

The study of these sera showed that they possessed high neutralizing power for the hemolytic poison obtained from the bodies of pneumococci, even higher than that present in the serum of rabbits or sheep injected with the toxin. This antihemolytic power, however, is not very specific as regards type of organisms, serum I protecting against the toxin prepared from organisms of type II almost as well as against that prepared from organisms of type I and *vice versa.* That this protective action is not merely a non-specific reaction of all immune sera, however, is shown by the fact that an anti-influenzal serum, kindly supplied by Dr. Wollstein,[11] possessed little or no greater antihemolytic power than did normal serum. In the experiment above described, the anti-influenzal serum had a little greater effect than did normal horse serum, but it was no greater than that of other normal horse sera tested at other times.

(*d*) *Antihemolytic Action of the Serum of Patients Sick of Pneu-*

[11] Wollstein, M., *Jour. Exper. Med.*, 1911, xiv, 73.

TABLE VII.

Toxin 1.70 Prepared from Pneumococci of Type I.

Experiments of October 7, 1913.			Hemolysis.			
			Immune horse serum I.	Immune horse serum II.	Normal horse serum.	Anti-influenzal serum.
Toxin (2 hemolytic units) + serum 0.1 c.c.	½ hr. at 37° C.	Sheep corpuscles 0.5 c.c.	o	o	+	o
Toxin (2 hemolytic units) + serum 0.02 c.c.		Sheep corpuscles 0.5 c.c.	o	o	+	±
Toxin (2 hemolytic units) + serum 0.01 c.c.		Sheep corpuscles 0.5 c.c.	o	o	+	+
Toxin (2 hemolytic units) + serum 0.002 c.c.		Sheep corpuscles 0.5 c.c.	o	ø	+	+
Toxin (2 hemolytic units) + serum 0.001 c.c.		Sheep corpuscles 0.5 c.c.	o	±	+	+
Toxin (2 hemolytic units) + serum 0.0002 c.c.		Sheep corpuscles 0.5 c.c.	+	+	+	+
Toxin A69, Prepared from Pneumococci of Type II.						
Toxin (2 hemolytic units) + serum 0.1 c.c.	½ hr. at 37° C.	Sheep corpuscles 0.5 c.c.	o	o	+	o
Toxin (2 hemolytic units) + serum 0.02 c.c.		Sheep corpuscles 0.5 c.c.	o	o	+	+
Toxin (2 hemolytic units) + serum 0.01 c.c.		Sheep corpuscles 0.5 c.c.	o	o	+	+
Toxin (2 hemolytic units) + serum 0.002 c.c.		Sheep corpuscles 0.5 c.c.	o	o	+	+
Toxin (2 hemolytic units) + serum 0.001 c.c.		Sheep corpuscles 0.5 c.c.	ø	±	+	+
Toxin (2 hemolytic units) + serum 0.0002 c.c.		Sheep corpuscles 0.5 c.c.	+	+	+	+

The tubes were kept at 37° C. for 1 hr. and 24 hrs. on ice.

monia and of Those Convalescent from That Disease.—The sera of patients suffering with pneumonia and those of patients during convalescence from this disease have been tested for antihemolytic power against the pneumococcus toxin. It has been impossible, however, to demonstrate that these sera possess an increased antihemolytic action over the controls with normal human serum and the serum of patients suffering from other diseases.

From these studies of the antihemolytic action of immune sera, it is evident that by the injection into rabbits and sheep of a solution containing the bacterial substance of pneumococci, the serum of these animals acquires an increased power of inhibiting the hemolytic action of such a solution. This change in the serum occurs

when the fluid injected consists of solutions of the bacterial bodies in sodium cholate or of solutions prepared by freezing the bacteria and grinding them in salt solution. The antihemolytic power of these sera is not so great, however, as that of sera produced by the injection of living organisms. The latter sera, however, possess marked agglutinating properties, while the former sera have no power of agglutination. While in the production of antitoxic sera the possibility of the injection of a few living organisms cannot be excluded, the lack of agglutinating power renders it extremely probable that the development of antihemolytic properties is due to the injection of the hemolytic substance, and that, therefore, this hemolytic solution possesses antigenic properties and may be considered a true toxin.

SUMMARY.

Solutions of the bodies of pneumococci, obtained by dissolving them in dilute solutions of sodium cholate, by permitting them to undergo autolysis, or by first freezing, drying, and then grinding in salt solution, are actively hemolytic for rabbit, sheep, guinea pig, and human red blood corpuscles. The substance on which this hemolytic property depends is very labile, much of its activity is lost on passing through a filter, and it is destroyed by the action of trypsin. In its properties it corresponds to the substance contained in such solutions which causes the death of guinea pigs on intravenous injection. Its activity is prevented by the presence of minute amounts of cholesterin.

Following the injection of this solution into rabbits and sheep, the sera of these animals acquire increased power of inhibiting its hemolytic action. It therefore possesses antigenic properties.

It may therefore be concluded that the bodies of pneumococci contain a toxin that is hemolytic for red blood corpuscles. This substance is not simply a product of autolysis but undoubtedly exists preformed in the bacterial cell. However, it is not given up to the surrounding fluid as long as the bodies of the bacteria are intact. It may therefore be considered a hemolytic endotoxin.

THE PRODUCTION OF METHEMOGLOBIN BY PNEUMOCOCCI.*

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When pneumococci are grown in media containing blood or hemoglobin, the red color of the latter is changed to a greenish brown, as may be well seen in blood agar plates on which pneumococci are growing. This change is undoubtedly due to the formation of methemoglobin. Butterfield and Peabody[1] have demonstrated that methemoglobin is formed when pneumococci are cultivated in media containing blood.

A similar change in color, though not so pronounced, is seen in the blood of animals dying of acute pneumococcus septicemia, and to a still less extent in the blood of patients severely ill and dying of pneumonia. Peabody[2] has shown that during the terminal stages of fatal cases of pneumonia there occurs a progressive decrease in the oxygen content and the oxygen-combining capacity of the blood. This is evidently due to the transformation of oxyhemoglobin into methemoglobin. A similar change occurs in the blood of rabbits severely infected with pneumococci.[3] While the presence of methemoglobin in artificial culture media may be readily demonstrated by spectroscopic methods, this is more difficult in the blood of patients and infected animals, since a considerable concentration of methemoglobin is necessary for spectroscopic demonstration.

It has seemed of importance to learn more of the nature of the reaction during which methemoglobin is formed by pneumococci, and the present paper gives a report of this study, as far as it has been carried out.

Peabody stated that the transformation of oxyhemoglobin into

* Received for publication, August 1, 1914.

[1] Butterfield, E. E., and Peabody, F. W., *Jour. Exper. Med.*, 1913, xvii, 587.

[2] Peabody, F. W., *Jour. Exper. Med.*, 1913, xviii, 7.

[3] Peabody, F. W., *Jour. Exper. Med.*, 1913, xviii, 1.

methemoglobin is brought about by the filtrates of pneumococcus cultures as well as by the cultures containing bacteria; but our experiments, which have been repeated many times, make it evident that in the experiments on which this statement was based faulty filters were employed. If the bacteria are all removed from a culture fluid, either by centrifugalization or by filtration through a Berkefeld filter, and then blood be added to the filtrate, no formation of methemoglobin occurs. The reaction therefore cannot depend merely upon the production of an acid reaction in the fluid in which the bacteria grow. This is also shown by careful neutralization of the culture before the addition of blood, when the rate of reaction is not changed unless to be increased.

Boiling a broth culture, or even heating it to 56° C. for one half hour, before addition of blood prevents the reaction from occurring. That this inhibition of the reaction by heat depends upon the destruction of the bacteria is rendered probable by the following experiment. A series of tubes containing broth cultures of different races of pneumococci were heated one half hour at 45° C.,—about the thermal death point for most races of pneumococci. Blood corpuscles were then added to each tube and transplantations were made from each tube on fresh media. In the cultures from which growth in the transplantation was obtained the formation of methemoglobin occurred. In the cultures, on the other hand, from which no growth occurred and in which the bacteria were therefore all killed, no formation of methemoglobin took place.

While extracts of pneumococcal bodies cause lysis of red blood corpuscles,[4] they never cause the formation of methemoglobin, unless they contain living pneumococci. The fact that small amounts of sodium cholate added to broth cultures of pneumococci inhibit the formation of methemoglobin might explain the failure of extracts of pneumococci in cholate solution to produce methemoglobin, but a similar failure to form methemoglobin is seen when the extracts are prepared by freezing and grinding the bacteria without the addition of cholate.

These experiments indicate that for the formation of methemoglobin the presence of living bacteria is necessary.

[4] Cole, R., *Jour. Exper. Med.*, 1914, xx, 346.

TABLE I.

Tube No.	Experiments.	Methemoglobin formation.	
		1 hr. at 37° C.	1 hr. at 37° C. and 24 hrs. on ice.
1	1 c.c. broth culture A8/0/6 + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	+ in 20 min.	+
2	1 c.c. broth culture A8/0/6 (1 : 2) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	+ in 20 min.	+
3	1 c.c. broth culture A8/0/6 (1 : 4) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	+ in 20 min.	+
4	1 c.c. broth culture A8/0/6 (1 : 8) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	+ in 20 min.	+
5	1 c.c. broth culture A8/0/6 (1 : 16) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	0	0
6	1 c.c. emulsion A + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	+ in 20 min.	+
7	1 c.c. emulsion A (1 : 2) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	+ in 20 min.	+
8	1 c.c. emulsion A (1 : 4) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	+	+
9	1 c.c. emulsion A (1 : 8) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	+	+
10	1 c.c. emulsion A (1 : 16) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	0	0
11	1 c.c. emulsion B + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	0	0
12	1 c.c. emulsion B (1 : 2) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	0	0
13	1 c.c. emulsion B (1 : 4) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	0	0
14	1 c.c. emulsion B (1 : 8) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	0	0
15	1 c.c. emulsion B (1 : 16) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	0	0

It was now important to learn whether or not washed pneumococci in salt solution could produce the reaction or whether the presence of nutrient medium also is required. That the latter is necessary is shown by the protocol of an experiment which follows. Two portions of a twenty-hour broth culture of pneumococcus A8/o/6 were centrifugalized, the supernatant fluid was removed, and each sediment thoroughly washed in sodium chloride solution and again centrifugalized. One portion of the sediment was now made up to the original volume in broth (emulsion A), and the other portion was made up to the original volume in sodium chloride solution (emulsion B) and tests were made to determine the methemoglobin-producing power (table I) of the two solutions in varying dilutions, compared with the methemoglobin-producing power of an untreated broth culture.

It was suggested that the failure of the reaction to occur in salt solution might be due to the toxicity of sodium chloride in the absence of other inorganic salts. To test this, experiments were carried out with organisms suspended in Ringer solution. No reaction, however, occurred. It is evident, therefore, that some organic constituents of the broth are necessary for the reaction.

Experiments were then made to determine which class of organic substances in broth is essential. The following is a protocol of one such experiment (table II).

While experiments like those in table II showed that the reaction occurs when either sugar, peptone, or protein is present, further studies have shown that while protein substances must be present in quite high concentration in order that the reaction may occur, at least one part of a 10 per cent. solution of crystallized egg albumin to twenty parts of salt solution being required, the reaction occurs with great rapidity when sugar in very great dilution is present, even in dilutions as great as one part of a 5 per cent. dextrose solution to 10,000 parts of salt solution. The presence of traces of sugar in peptone solution and even in solutions of egg albumin can only with great difficulty be excluded, and it is possible that upon the presence of such traces of sugar the availability of these solutions in this reaction depends, since only extremely small amounts of sugar are required.

Various kinds of sugar were next tested to determine whether

TABLE II.

Tube No.	Experiments of December 13, 1913.	Methemoglobin formation.	
		1 hr. at 37° C.	24 hrs. on ice.
1	1.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	0	0
2	1 c.c. sterile broth + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
3	1 c.c. sterile broth (1 : 2) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
4	1 c.c. sterile broth (1 : 4) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
5	1 c.c. sterile broth (1 : 8) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
6	1 c.c. sterile broth (1 : 16) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
7	1 c.c. 5% dextrose + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
8	1 c.c. 5% dextrose (1 : 2) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
9	1 c.c. 5% dextrose (1 : 4) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
10	1 c.c. 5% dextrose (1 : 8) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
11	1 c.c. 5% dextrose (1 : 16) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
12	1 c.c. 5% peptone + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
13	1 c.c. 5% peptone (1 : 2) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
14	1 c.c. 5% peptone (1 : 4) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
15	1 c.c. 5% peptone (1 : 8) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
16	1 c.c. 5% peptone (1 : 16) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
17	1 c.c. 4% egg albumin + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
18	1 c.c. 4% egg albumin (1 : 2) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
19	1 c.c. 4% egg albumin (1 : 4) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
20	1 c.c. 4% egg albumin (1 : 8) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
21	1 c.c. 4% egg albumin (1 : 16) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
22	1 c.c. sterile broth + 1 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution	0	0
23	1 c.c. 5% dextrose + 1 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution	0	0
24	1 c.c. 5% peptone + 1 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution	0	0
25	1 c.c. 4% egg albumin + 1 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution	0	0

The hemoglobin solution = 2 c.c. washed sheep corpuscles plus 8 c.c. distilled water plus 30 c.c. 0.85 per cent. sodium chloride solution.

Dextrose = 5 per cent. solution of chemically pure dextrose in distilled water, autoclaved for 20 minutes.

Peptone = 5 per cent. solution of Witte's peptone in distilled water, autoclaved for 20 minutes.

Egg albumin = 4 per cent. solution of crystallized egg albumin in distilled water.

Bacterial emulsion = emulsion in sodium chloride solution of washed sediment of 20-hour broth culture A8. The bacteria were in twice as great concentration as in the culture.

pneumococci cause the formation of methemoglobin only in the presence of dextrose or whether this sugar may be replaced by others in which the molecular configuration is different. Several races of pneumococci as well as streptococci were employed and the results are given in table III.

In another experiment *d*-xylose and *d*-arabinose were also tested. The results are given in table IV.

While in the experiments given no reaction occurred in the presence of inulin, in several other experiments a slight reaction occurred in solutions containing 2 per cent. of this sugar. In these and many other experiments as well, however, no reaction ever occurred in the solutions containing ribose. It is therefore apparent that while the reaction occurs in the presence of most sugars, the configuration of the molecule makes some difference. This is also seen in the effect of different sugars on the rate of reaction. The reaction always occurred more slowly with saccharose and arabinose than with the other sugars. Since the reaction occurs when pneumococci are placed in solutions containing traces of sugar, and it is known that pneumococci cause breaking down of many sugars, the possibility suggested itself that the reaction is due to some substance formed during the decomposition of the sugar molecule. The reaction cannot, however, be due simply to the formation of carbon dioxide. If carbon dioxide be passed through a solution of oxyhemoglobin the solution becomes cherry red, and if oxygen now be passed through it again becomes bright red. Such changes never occur in solutions of hemoglobin acted upon by pneumococci. A similar change is well seen when yeast is added to solutions containing blood corpuscles. In this case the blood takes on a magenta color which on shaking becomes bright red. It would appear possible, however, that the change might be due to some intermediate product of sugar metabolism. The exact transformation which the sugar molecule undergoes during the process of changing into carbon dioxide and water is still obscure, but there are several substances which are thought to represent intermediate stages. A series of such substances which are thought to be intermediate products in the metabolism of sugar and also certain ones that are known to be end products under certain conditions were tested. These substances

TABLE III.

Experiments.	Methemoglobin formation.			
	A18	P1.77	M1.77	Streptococcus.
1 c.c. 2% ribose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution.....	0	0	0	0
1 c.c. 2% arabinose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution....	+ in 1 hr.	+ in 10 min.	+ in 1 hr.	0
1 c.c. 2% xylose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution.....	+ in 10 min.	+ in 10 min.	+ in 10 min.	0
1 c.c. 2% maltose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution.....	+ in 1 hr.	+ in 10 min.	+ in 10 min.	0
1 c.c. 2% lactose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution.....	+ in 1 hr.	+ in 10 min.	+ in 10 min.	0
1 c.c. 2% saccharose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution ...	+ in 1 hr.	+ in 1 hr.	+ in 1 hr.	0
1 c.c. 2% dextrose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution....	+ in 10 min.	+ in 10 min.	+ in 10 min.	0
1 c.c. 2% levulose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution....	+ in 10 min.	+ in 10 min.	+ in 10 min.	0
1 c.c. 2% mannose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution....	+ in 10 min.	+ in 10 min.	+ in 10 min.	0
1 c.c. 2% galactose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution....	+ in 10 min.	+ in 10 min.	+ in 10 min.	0
1 c.c. 2% inulin solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution.....	0	0	0	0
1 c.c. 2% raffinose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution	+ in 10 min.	+ in 10 min.	+ in 10 min.	0

The tubes were all kept at 37° C.

A18, P1.77, and M1.77 are pneumococci of type I. The streptococcus is a virulent hemolytic streptococcus.

TABLE IV.

Experiments.	Methemoglobin formation.		
	A19/5/7	A26/0/3	1/73
1 c.c. ribose.....2 % solution + 1 c.c. bacterial emulsion + 0.5 c.c. hemoglobin solution	0	0	0
1 c.c. *l*-arabinose.....2 % solution + 1 c.c. bacterial emulsion + 0.5 c.c. hemoglobin solution	+ in 15 min.	+ in 15 min.	+ in 15 min.
1 c.c. *d*-arabinose.....2 % solution + 1 c.c. bacterial emulsion + 0.5 c.c. hemoglobin solution	0	0	0
1 c.c. *l*-xylose.....2 % solution + 1 c.c. bacterial emulsion + 0.5 c.c. hemoglobin solution	+ in 15 min.	+ in 15 min.	+ in 15 min.
1 c.c. *d*-xylose.....2 % solution + 1 c.c. bacterial emulsion + 0.5 c.c. hemoglobin solution	0	0	0
1 c.c. dextrose.....2 % solution + 1 c.c. bacterial emulsion + 0.5 c.c. hemoglobin solution	+ in 15 min.	+ in 15 min.	+ in 15 min.
1 c.c. mannose.....2 % solution + 1 c.c. bacterial emulsion + 0.5 c.c. hemoglobin solution	+ in 15 min.	+ in 15 min.	+ in 15 min.
1 c.c. inulin.....2 % solution + 1 c.c. bacterial emulsion + 0.5 c.c. hemoglobin solution	0	0	0

The tubes were kept for 2 hours at 37° C.

were kindly supplied by Dr. G. M. Meyer. They were methylglyoxal, acetaldehyde, pyruvic acid, formaldehyde, formic acid, and acetic acid. Methylglyoxal, acetaldehyde, formaldehyde, and pyruvic acid produced methemoglobin only when very concentrated solutions were employed. The presence of bacteria did not increase their effect. Since such extremely small amounts of sugar are required in the formation of methemoglobin by bacteria, it is not possible that under these conditions any of the substances are present in a concentration sufficient to bring about the reaction, unless such substances are much more active in a nascent state than they are in solution.

The tests with formic acid showed that this substance caused the formation of methemoglobin only when it was used undiluted or but slightly diluted. Even under these conditions the reaction is due to the acidity of the solution, since, after neutralization and the addition of a mixture of primary and secondary potassium phosphates, in order to keep the mixture neutral, no reaction occurred, even with the undiluted acid. When a solution of formic acid, not in itself able to produce methemoglobin, is added to the bacterial emulsion plus hemoglobin solution, no formation of methemoglobin occurs, while the addition of a very dilute solution of dextrose under similar conditions causes the reaction to occur within a few minutes.

Similar results were obtained when acetic acid was used.

These studies, therefore, did not lead to the detection of any substance formed during sugar metabolism or fermentation to which the change of oxyhemoglobin into methemoglobin could be ascribed.

Other substances, such as alcohols, were also tested as to their effect in the formation of methemoglobin. The following results were obtained (table V).

None of these substances, except glycerin, when employed in low dilutions are able to replace dextrose in the mixtures in which methemoglobin is formed. When, however, glycerin, even in a dilution of 1 to 10,000, is added, the formation of methemoglobin occurs rapidly.

A large number of quantitative studies with these and similar substances were made, in the hope of finding some relationship in chemical structure between those substances which by their presence per-

TABLE V.

Experiments.	Results.
2 c.c. glycerin ..... (10 % solution) + 0.5 c.c. hemoglobin solution	No change in 24 hrs.
2 c.c. mannite ..... (10 % solution) + 0.5 c.c. hemoglobin solution	No change in 24 hrs.
2 c.c. methyl alcohol ..... (10 % solution) + 0.5 c.c. hemoglobin solution	No change in 24 hrs.
2 c.c. ethyl alcohol ..... (10 % solution) + 0.5 c.c. hemoglobin solution	No change in 24 hrs.
1 c.c. bacterial emulsion + 1 c.c. glycerin ..... (1 : 10,000) + 0.5 c.c. hemoglobin solution	+ in 10 min.
1 c.c. bacterial emulsion + 1 c.c. mannite ..... (10 % solution) + 0.5 c.c. hemoglobin solution	+ in 3 hrs.
1 c.c. bacterial emulsion + 1 c.c. methyl alcohol ..... (10 % solution) + 0.5 c.c. hemoglobin solution	+ in 3 hrs.
1 c.c. bacterial emulsion + 1 c.c. ethyl alcohol ..... (10 % solution) + 0.5 c.c. hemoglobin solution	+ in 2 hrs.
1 c.c. bacterial emulsion + 1 c.c. dextrose ..... (5 % solution) + 0.5 c.c. hemoglobin solution	+ in 10 min.
1 c.c. bacterial emulsion + 1 c.c. starch ..... (1 % solution) + 0.5 c.c. hemoglobin solution	+ in 1 hr.
1 c.c. bacterial emulsion + 1 c.c. urea ..... (2 % solution) + 0.5 c.c. hemoglobin solution	No change in 24 hrs.
1 c.c. bacterial emulsion + 1 c.c. ethyl acetate ..... (0.2 % solution) + 0.5 c.c. hemoglobin solution	+ in 1 hr.
1 c.c. bacterial emulsion + 1 c.c. leucin ..... (2 % solution) + 0.5 c.c. hemoglobin solution	No change in 24 hrs.
1 c.c. bacterial emulsion + 1 c.c. cholesterin ..... (1 % solution) + 0.5 c.c. hemoglobin solution	No change in 24 hrs.

mit pneumococci to form methemoglobin. So large a number of substances are suitable for the purpose that it has not been possible to show that any form of molecular configuration or grouping is essential.

It seems probable, judging from these studies, that the formation of methemoglobin occurs whenever pneumococci in contact with hemoglobin are able to carry on metabolic and functional activities, probably including multiplication, and that this happens when traces of sugar and also when large amounts of other organic substances are present. While in the absence of those substances death of the bacteria does not necessarily occur, it is probable that their metabolic activities are reduced to a minimum.

Cellular metabolic processes occur through the agency of ferments contained within the cells, and it would therefore seem probable, if the reaction under consideration were due to the active processes of oxidation occurring in the medium immediately surrounding the bacterial cells, that similar reactions would appear when bacterial extracts are used instead of the living bacteria. As Warburg[5] has pointed out, however, the reactions which occur in the living cell are not identical, at least in intensity, with those induced by the ferments when removed from the cell by extraction or other means. The activity of zymase, for instance, is markedly less than that of a corresponding number of living yeast cells. The importance of structure in cellular activity cannot be neglected, and from the fact that the reaction under consideration is not induced by the presence of bacterial extracts, it does not necessarily follow that the reaction is not due to the functional activities of the bacterial cells.

Studies concerning the formation of methemoglobin from oxyhemoglobin by means of chemicals indicate that the reaction is of the nature of an oxidation. The exact chemical nature of oxyhemoglobin as well as that of methemoglobin has not been absolutely determined. It is not even definitely known whether the methemoglobin molecule contains an amount of oxygen equal to or less than that of the oxyhemoglobin molecule. There is considerable evidence,[6] however, for the view that while in oxyhemoglobin the

[5] Warburg, O., Ueber die Wirkung der Struktur auf chemische Vorgänge in Zellen, Jena, 1913.

[6] von Reinhold, B., *Ztschr. f. physiol. Chem.*, 1913, lxxxv, 250.

oxygen is loosely combined as in an oxide, the formula being usually written $Hb\langle^{O}_{O}$, in methemoglobin the oxygen is as in an hydroxide. Whether the molecule contains one hydroxyl group as in Hb–OH, or two hydroxyl groups as in $Hb\langle^{O\ H}_{O\ H}$, is not determined, though there is experimental evidence for both points of view. In either case, however, methemoglobin would represent a lower stage of oxidation than oxyhemoglobin, and the transformation of oxyhemoglobin into methemoglobin would therefore be of the nature of a reduction.

On the other hand evidence has been presented by Heubner[7] to show that the formation of methemoglobin is always an oxidation process. As is well known, substances which are known to be oxidizing agents and also those considered to be reducing agents may bring about the transformation of oxyhemoglobin into methemoglobin. An explanation of this fact, which Heubner presents, is that the reducing agents are first oxidized, this oxidation occurring better in the presence of oxyhemoglobin, and then reduced, giving up their oxygen to form methemoglobin. It is of great interest that certain substances, such as aminophenol, $NH_2\langle\rangle OH$, are able to convert much more hemoglobin into methemoglobin than could occur if the reaction were a simple molecular one. One molecule of aminophenol may apparently transform at least fifty molecules of hemoglobin into methemoglobin. The aminophenol must therefore repeatedly react with the hemoglobin. Heubner's view is that the aminophenol is first oxidized to a quinone, (quinonimine, $NH\langle\rangle O$) and then again reduced to aminophenol, in this reaction the hemoglobin being changed to methemoglobin. The reaction may be repeated many times, the aminophenol therefore having a catalytic-like action.

If this be the true explanation of the kind of reaction occurring in the transformation of oxyhemoglobin into methemoglobin by organic chemical substances, it is possible that the mode of action of pneumococci in causing the formation of methemoglobin may fol-

[7] Heubner, B., *Arch. f. exper. Path. u. Pharmakol.*, 1913, lxxii, 239.

low similar lines. The metabolic activities of cells consist largely of oxidative and reduction processes. When oxyhemoglobin is brought into contact with pneumococci it may be changed into methemoglobin, because it is then exposed to active oxidative and reduction processes occurring in the neighborhood of the bacterial cells. These processes must be of a special type in the case of pneumococci, however, since otherwise all living cells would cause the transformation.

That this change goes on in the neighborhood of the cells and not necessarily in the cells themselves is shown by the fact that while the reaction occurs more readily when the hemoglobin is in solution in the medium surrounding the bacteria, it may also occur when the hemoglobin is contained within red blood corpuscles, the blood cells and bacteria being merely in intimate contact. If, however, the bacteria and blood cells are separated by a membrane of any kind, even a very thin layer of oil, the reaction does not occur.

If the change of hemoglobin into methemoglobin taking place in the neighborhood of pneumococcal cells is due to oxidative processes occurring there, it was thought that evidence for this fact would be obtained by studying the effect which the presence or absence of free oxygen would have on the reaction. This was done by testing the rate of reaction under the following conditions: To remove oxygen from a solution, a stream of hydrogen was passed through it for ten minutes, the entrance tube passing completely to the bottom of the tube containing the solution. The solution was then covered by a layer of paraffin oil. Passing hydrogen in this way through a solution of hemoglobin causes the solution to take on a dark color due to the formation of reduced hemoglobin. In mixing two solutions so treated, a pipette was passed through the paraffin layer of one to the bottom of the tube and the fluid was drawn into the pipette and added to the second solution, by plunging the tip of the pipette through the oil on the surface. Care was taken not to empty the pipette completely, as in this way air would be admitted. While complete absence of oxygen could not be obtained by this method, it was sufficiently excluded for the purpose intended.

It was found that if an emulsion of red blood cells, so treated with hydrogen, was added to a broth culture of pneumococci, also treated with hydrogen, no formation of methemoglobin occurred for some hours. When the tubes stood over night there usually

occurred some change into methemoglobin, usually only at the surface under the layer of oil. If, moreover, such a mixture has stood for some time without the formation of methemoglobin, and oxygen now be bubbled through, the change into methemoglobin occurs with great readiness. It is evident, therefore, that the presence of oxygen is necessary for the reaction.

On the other hand, an excess of free oxygen somewhat delays the reaction. If two tubes are prepared, each containing a mixture of an emulsion of red blood cells and an emulsion of pneumococci, and if oxygen be bubbled through one tube, while the other is simply allowed to stand exposed to the air, the two tubes being kept at the same temperature, the change into methemoglobin occurs more slowly in the tube through which oxygen is passing. To control the possibility that the mechanical disturbance due to the bubbling gas may account for the difference, air was passed through the second tube. The reaction was again delayed in the tube through which oxygen was passing.

Two tubes were now prepared, one tube containing a mixture of an emulsion of red blood cells and an emulsion of bacteria, each of which had been previously saturated with hydrogen, and the other containing an identical mixture except that no hydrogen had been passed through. Oxygen was now bubbled through both tubes, which were kept at the same temperature and under identical conditions. In the tube containing the mixture previously saturated with hydrogen the reaction occurred more rapidly than it did in the other tube.

The above results are briefly shown in the following protocol of one experiment (table VI).

It is now easy to interpret these experiments in the light of

TABLE VI.

Tube No.	Experiments.		Methemoglobin formation.
1	4 c.c. broth culture A82/1/12 + 2 c.c. hemoglobin solution.....		+ in 7 min.
2	4 c.c. broth culture A82/1/12 saturated with hydrogen + 2 c.c. hemoglobin solution saturated with hydrogen		0
3	4 c.c. broth culture A82/1/12 + 2 c.c. hemoglobin solution	Oxygen bubbled through	+ in 28 min.
4	4 c.c. broth culture A82/1/12 saturated with hydrogen + 2 c.c. hemoglobin solution saturated with hydrogen	Oxygen bubbled through	+ in 17 min.

The tubes were all kept at 37° C.

what is known concerning the mode of production of methemoglobin by substances like aminophenol. According to this interpretation the formation of methemoglobin by pneumococci occurs as the result of reduction and oxidative processes occurring in the neighborhood of the bacteria. The oxyhemoglobin is first reduced and if this is inhibited by an excess of free oxygen the reaction is delayed. On the other hand, after reduction has occurred a free supply of oxygen accelerates the reaction. If oxygen be excluded no reaction whatever can occur.

The writer realizes that with the present knowledge it is impossible to conclude that precisely this mode of reaction occurs; but it seems to be the best explanation at present available of the observed experimental facts. It may be objected that the effect of the presence of hydrogen and oxygen is to inhibit or accelerate the metabolic activities of the bacteria rather than to cut off or to increase the supply of oxygen required for the chemical changes. The fact that an excess of oxygen delays the reaction is against this interpretation, though it is known that an excess of oxygen may inhibit cellular action or even be directly toxic to cells.

Experiments were also undertaken to determine whether, in the absence of free oxygen, the oxygen required for the reaction could be obtained from methylene blue, if this be added to the mixture. It was found that the reaction proceeds under these circumstances, but more slowly and less completely than in the presence of free oxygen.

It is believed that the experiments concerning the production of methemoglobin by pneumococci are important not only because they may possibly explain a reaction which probably occurs in every animal severely infected with pneumococci, but they are also important because they suggest a possible explanation for the pathological action of those bacteria which apparently do not produce an active toxin. Since bacteria may injure red blood corpuscles by merely changing oxidative processes in their vicinity, and without producing substances capable of isolation, it is possible that bacteria may injure other tissue cells in a similar manner. Therefore, the pathological effects of bacteria are not necessarily due to the action of a definite poison, but may be due to disturbances in oxidation in the immediate neighborhood of the bacteria.

CONCLUSIONS.

1. Pneumococci in contact with hemoglobin transform this into methemoglobin. This reaction occurs only when the pneumococci are living; it is not induced by the culture fluid or by extracts of the bacteria.

2. The reaction does not occur when hemoglobin is added to an emulsion of washed pneumococci in salt solution. However, if minute traces of dextrose be added to such a mixture, the reaction quickly occurs. The dextrose may be replaced by any one of a number of other sugars, and also by certain other organic substances, if the latter are added in large amounts. Certain other organic substances are not able to replace dextrose, but it has been impossible to determine any special molecular configuration on which this property depends.

3. The formation of methemoglobin by pneumococci probably resembles the formation of methemoglobin by certain chemical substances, such as aminophenol.

4. From the work of others it is probable that the formation of methemoglobin is always a reaction of oxidation. In the formation of methemoglobin by reducing agents, the latter are first oxidized, this occurring better in the presence of oxyhemoglobin. In certain instances an alternate oxidative and reduction of the transforming agent occurs, so that the reaction is continuous.

The effect which the presence or absence of free oxygen has on the reaction with pneumococci suggests that this follows similar lines.

5. The reaction does not occur in the absence of oxygen. If the free oxygen be first removed, and then replaced, the reaction occurs more rapidly than if the oxygen had not been removed. The presence of free oxygen in excess slightly delays the reaction, possibly because of the inhibition of the reduction process which forms the first part of the reaction.

6. The explanation of this phenomenon of methemoglobin production is not only of importance so far as this special reaction is concerned, but also because it suggests an explanation for the manner in which pathological effects are produced by those bacteria which apparently produce no soluble toxin.

THE RÔLE OF THE SPLEEN IN BLOOD FORMATION.*

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Various authors[1] have described the appearance in the spleen in the course of chronic anemias of abundant cells which give evidence of the new formation of blood in that organ when blood formation is greatly needed by the body. These cells are erythroblasts and mononuclear cells of various sizes which become very abundant in the venous sinuses. The same cells are found in the capillaries of the liver and occasionally outside them in the periportal tissues, but not in great numbers in the circulating blood.

The information derived from attempting to estimate the change in the numbers of cells in such an organ as the spleen was felt to be unsatisfactory and a method of study other than the staining of sections of the fixed spleen was sought. Without any knowledge of previous efforts in this direction it was thought that accurate counts of the blood leaving the spleen by the splenic vein when compared with similar counts of that entering by the splenic artery should give a clear idea of the activity of the spleen with respect to its contribution of cells to the blood. Since the completion of the work a few references to similar studies have been found, but it nevertheless seems that a detailed statement of the result of these counts would be of value, especially since the method which is so useful has been employed by hardly any modern workers.

* Received for publication, August 5, 1914.

[1] Bizzozero, G., and Salvioli, G., *Arch. p. le sc. med.*, 1880, iv, 49. Meyer, E., and Heineke, A., *Verhandl. d. deutsch. path. Gesellsch.*, 1906, ix, 224; *Deutsch. Arch. f. klin. Med.*, 1907, lxxxviii, 435. Morris, R. S., *Bull. Johns Hopkins Hosp.*, 1907, xviii, 200. von Domarus, A., *Arch. f. exper. Path. u. Pharmakol.*, 1908, lviii, 319. Heinz, R., *Virchows Arch. f. path. Anat.*, 1902, clxviii, 501. Jolly, J., and Rossello, H., *Compt. rend. Soc. de biol.*, 1909, lxvi, 40. Schridde, H., *Centralbl. f. allg. Path. u. path. Anat.*, 1908, xix, 865. Naegeli, O., Ehrlich, P., and Lazarus, A., Die Anaemie, Vienna and Leipzig, 1909.

It has been known for a long time that the leucocytic count of the blood from the splenic vein exceeds that of the artery.

Vierordt[2] in four counts made on an executed man found the relation of leucocytes to red corpuscles in the splenic vein to be 1 : 49. Hirt[3] found that in the calf the relation was 1 : 60, while in the arterial blood it is 1 : 2,200. Funke[4] gives this relation in the splenic vein as 1 : 4, while Frey[5] calculated the proportion in an old man dead of pneumonia as 1 : 102. Rindfleisch[6] called attention to the richness of the splenic blood in leucocytes especially after a meal. Weidenreich[7] undertook counts from the sinuses of the spleen and obtained variable results inasmuch as some of them contained few or no leucocytes, while others were filled with them doubtless because those sinuses into which the arterial stream enters directly will contain predominantly red corpuscles while the reverse condition will obtain where the lymph channels enter. As a mean I found a proportion of 1 : 15. If we take the relation in the artery (following Hirt) as 1 : 2,200, and the average of the figures from the literature as 1 : 30 it is seen that the splenic vein contains about seventy times as many leucocytes as the efferent artery. Since special efferent lymphatic channels for the drainage of the lymphocytes of the spleen have been shown not to exist, the cells must be discharged into the vena lienalis which thus contains both blood and lymph.

Von Ebner[8] states that it has been shown that the blood of the splenic vein contains an uncommonly large number of white corpuscles and that the site of their formation is the splenic tissue itself from which they enter through the permeable walls of the venules. Loewit[9] studied the relation of leucocytes of arterial blood and splenic venous blood in the guinea pig and found 30 to 80 times as many leucocytes in the venous blood, the increased proportion being due to small and large mononuclear elements. This finding is not obtained in all animals probably because of the irregular or intermittent discharge of the cells. Certain negative results of Tarchanoff and Swaen[10] are probably due to this cause.

The cardinal idea, therefore, in the present study was to examine carefully the blood entering the spleen and to compare it with that emerging by the splenic vein. Any differences should

[2] Vierordt, *Arch. f. physiol. Heilk.*, 1854, xiii, 259, 408.

[3] Hirt, E., *Arch. f. Anat., Physiol., u. wissensch. Med.*, 1856, 174.

[4] Funke, O., Lehrbuch der Physiologie, Leipzig, 1863, i.

[5] Frey, H., Handbuch der Histologie und Histochemie des Menschen, 4th edition, Leipzig, 1874.

[6] Rindfleisch, G. E., Experimentelle Studien ueber die Histologie des Blutes, Leipzig, 1863.

[7] Weidenreich, F., *Arch. f. mikr. Anat.*, 1901, lviii, 247.

[8] von Ebner, V., *Köllikers Handbuch der Gewebelehre*, 1902, iii, 276.

[9] Loewit, M., *Folia Haematol.*, 1907, iv, 473.

[10] Tarchanoff, J., and Swaen, A., *Arch. de physiol. norm. et path.*, 1875, ii, series 2, 324.

represent the changes produced by the spleen. Counts were made with the Thoma-Zeiss apparatus with blood from the splenic artery, splenic vein, mesenteric vein, and, for purposes of control, from a peripheral systemic vein. Smears of blood were also made from each situation. Rabbits, cats, and dogs were studied, some in perfect health, others suffering from spontaneous or artificially induced disease. Thorough autopsies were made in each case and the organs studied microscopically. The counts were made in the usual way but with the most scrupulous attention to technical accuracy in every detail. For the white corpuscles 0.2 per cent. acetic acid was used and for the red cells fresh Toison's solution. Differential counts of the white cells were made in all cases for the blood from each situation.

In securing blood from the splenic artery and vein considerable care must be taken to have a dry field without great loss of blood in the earlier steps of the operation. An incision from the midline along the left costal margin to the axillary line was found to give the best exposure, after which the assistant could elevate the spleen without making traction upon the vessels, by lifting up the stomach and gently drawing the spleen forward so as to expose the hilum. Care was taken not to handle or squeeze the spleen so as to force out its contents artificially. The remaining viscera were protected and the spleen supported by warm cloths so that the circulation proceeded normally. The blood must be obtained from the splenic vein first, as interference with the arterial current will produce changes in that of the veins, while the venous anastomosis and collateral trunks are so numerous that occlusion of one produces no effect.

Some counts were made from blood allowed to spurt from a puncture in the vein, but since this hemorrhage makes it difficult to work later with the artery it was found advisable to put on two Carrel clamps and make the puncture between them. The latter is the more satisfactory procedure since a few drops only may be allowed to flow, and the counts do not differ appreciably from those made from the freely flowing blood.

Ehrlich's triacid stain after fixation by heat and Jenner's, Wright's, and Giemsa's stains after methyl alcohol fixation were

used for the differential counts. Prolonged staining with diluted Giemsa's stain gave good results.

The experiments showed that the blood of the splenic vein differs remarkably from that of the artery not only in the character of its cells but in their number, and this difference was found to exist also between the blood of the splenic vein and that of a peripheral systemic vein.

These differences may be summed up as follows:

(1) The number of red corpuscles per cubic millimeter in the blood of the splenic vein is greater than in that of the artery.

(2) Similarly the number of white corpuscles in the venous blood is greater than in the arterial blood.

(3) The character and proportion of the several varieties of white corpuscles is different in the splenic vein from what is found in the artery. Large mononuclear leucocytes appear in great excess there.

(4) The blood of the splenic vein contains more perfect and larger red corpuscles than that of the artery and they seem richer in hemoglobin.

(5) The blood of the inferior mesenteric veins differs from that of the splenic vein in being relatively richer in small mononuclear cells and poorer in the large mononuclears.

In the tabulated and detailed results given below (table I) these differences are more definitely brought out. It will be noted that the counts made from the ear vein and splenic artery of the same animal correspond very closely, as would be expected, while a marked difference was always found when blood was taken from the splenic veins.

It will be seen from table I that when the autopsy showed the animals operated on to be normal the results were invariably the same. There were always more red and white cells in the splenic veins than could be found in any of the other vessels. Frequently this excess of corpuscles rose to such a degree that there were twice as many red or white cells coming out as going into the spleen. That the excess of white cells should be mainly of the mononuclear variety was to be expected. But there occurred a further noteworthy fact, namely, that these mononuclears were mainly of the

TABLE I.

Animal.	Blood count.	Ear vein.	Splenic artery.	Splenic vein.	Inferior mesenteric vein.	Autopsy.
Rabbit 1	Red blood count	5,400,000	5,200,000	6,700,000		Small nodule of coccidiosis in liver, otherwise normal.
	White blood count	4,000	4,000	9,400		
	Polynuclears	60%	61%	32%		
	Mononuclears	40%	39%	68%		
Rabbit 2	Red blood count	5,900,000	5,700,000	8,600,000		All organs appear normal.
	White blood count	10,600	10,000	25,600		
	Polynuclears	47%	49%	20%		
	Mononuclears	53%	51%	80%		
Rabbit 3	Red blood count	4,600,000	4,560,000	5,360,000		Young rabbit, normal.
	White blood count	5,200	5,000	13,600		
	Polynuclears	30 to 32%	30 to 32%	18 to 20%		
	Mononuclears	68 to 70%	68 to 70%	80 to 82%		
Rabbit 4	Red blood count	Not done	5,280,000	6,800,000		Lactating rabbit. Marked coccidiosis in liver. Spleen small, shrunken, and dark red.
	White blood count	9,800	9,400	4,600	Portal vein	
	Polynuclears	38%	36%	10%	32%	
	Mononuclears	62%	64%	90%	68%	
Dog 1	Red blood count	5,280,000	5,280,000	6,640,000		Normal.
	White blood count	17,000	16,400	34,400	16,400	
	Polynuclears	90%	85%	70%		
	Mononuclears	10%	15%	30%		
Dog 2	Red blood count	4,500,000	4,320,000	6,600,000		Normal.
	White blood count	6,900	7,000	14,800	10,600	
	Polynuclears	61%	60%	44%	45%	
	Mononuclears	39%	40%	56%	55%	
	Large mononuclears			47%	10%	
	Small mononuclears			9%	45%	
Dog 3	Red blood count	5,900,000	5,800,000	7,240,000		All organs normal.
	White blood count	19,700	19,700	19,900		
Dog 4	Red blood count	4,200,000	4,100,000	6,500,000		All organs normal.
	White blood count	20,000	20,000	25,000		
	Polynuclears	80%	81%	70%		
	Mononuclears	20%	19%	30%		
	Large mononuclears	12%	12%	24%		
Cat 1	Red blood count		4,400,000	9,120,000		Apparently normal.
	White blood count		18,000	44,000		
	Polynuclears		75%	30%		
	Mononuclears		25%	70%		

TABLE I.—*Continued.*

Animal.	Blood count.	Ear vein.	Splenic artery.	Splenic vein.	Inferior mesenteric vein.	Autopsy.
Cat 2	Red blood count		9,600,000	13,104,000	9,440,000	All organs apparently normal except spleen which is enlarged to about 4 times its normal size and is very mottled on cross-section, due to tremendously enlarged Malpighian bodies. Two accessory spleens presenting same structure. *Microscopical Examination.*—Enormously enlarged follicles. Active mitoses. Huge mononuclear cells.
	White blood count		89,400	137,200	89,400	
	Polynuclears		75 %	74 %	75 %	
	Mononuclears		25 %	26 %	25 %	
	Large mononuclears		25 %	25 %	20 %	
	Small mononuclears		0	1 %	5 %	
Cat 3	Red blood count		7,280,000	11,200,000		Spleen appears normal in size, color, shape, and cross-section. Liver normal. All other organs except mesenteric lymph nodes are normal. Mesenteric lymph nodes especially in duodenal region are enormously enlarged, firm, and matted together in places. Dark red in color. *Microscopical Examination.*—Sections of lymph nodes show tremendous enlargement of germinal centers. Long rod-like bacilli in marginal sinuses. Sections of spleen and liver normal.
	White blood count		26,000	13,500	26,000	
	Polynuclears		18%	40%	19%	
	Mononuclears		82%	60%	81%	
	Large mononuclears		11%	50%	9%	
	Small mononuclears		71%	10%	72%	

TABLE I.—*Concluded.*

Animal.	Blood count.	Left ventricle.	Right auricle.	Splenic vein.	Portal vein.	Autopsy.
Received intraperitoneal inoculation of virulent paratyphoid culture 24 hrs. previously.						
Rabbit 5	Red blood count	4,300,000		6,240,000		Peritonitis. Focal necroses in liver. Large mitotic cells in spleen, especially at margins of follicles.
	White blood count	450		7,500		
	Polynuclears		40%	12%	88%	
	Mononuclears		60%	88%	12%	

large variety, whereas those coming from the adenoid tissue of the intestines and mesenteric nodes were mainly of the small variety. The significance of this was clearly shown in cat 3, where the white cells coming from the spleen were found to be only half as numerous as in the peripheral circulation and in the inferior mesenteric vein. The autopsy showed the reason for this. The spleen was unchanged and discharged its normal quota of large mononuclears into the blood stream; but the adenoid tissue in the mesentery was enormously hypertrophied and was discharging great numbers of small lymphocytes into the circulation. Microscopic sections showed the efferent vessels of these nodes to be packed with small mononuclears.

One of the most striking evidences of splenic blood formation was that of cat 2, in which 13,000,000 red cells and 137,000 white cells per cubic millimeter were found in the splenic vein as against about two thirds of that number in the peripheral circulation. The reason for this at once became apparent on examination of the spleen itself. There was an enormous hyperplasia of the organ, and the germinal centers on microscopical section showed very active production of large mononuclears. There were furthermore two accessory spleens. The organs elsewhere were normal and the cat seemed in good health.

Another marked instance of the difference between splenic and portal corpuscular content was seen in dog 2, in which the proportions of large and small mononuclears were almost exactly the reverse in the two veins.

Most interesting were the results in rabbit 5, which had been previously inoculated with a rather virulent strain of paratyphoid

bacillus. This strain in rabbits causes a marked leucopenia in the systemic vessels as a constant feature following inoculation. But the remarkable thing was that although this leucopenia had reached such a grade that the heart's blood contained only 450 white blood cells to the cubic centimeter the spleen still kept discharging leucocytes at a rate of 7,500 per cubic centimeter, a perfectly evident effort at compensation for the loss in the general blood stream.

These results all point to the inevitable conclusion that the spleen is a blood-forming organ of prime importance in the animal metabolism. The fact that the organ can be extirpated without causing death or even considerable detriment to the animal organism does not militate against this conclusion. Other organs (hemolymph nodes, bone marrow, and adenoid tissues in general) may assume part of the rôle of the spleen when this is absent, but only the severity of the blood-destroying agent and the individual resistance can determine whether the body can stand the strain when deprived of the spleen. Cases of death from removal of the malarial spleen indicate this strongly. And this same individual variation will doubtless account for the discrepancy in the results obtained by different observers regarding the importance of the spleen in the animal economy.

In closing, I desire to express my gratitude to Dr. William G. MacCallum for his interest in this work.

ON THE IMMUNIZATION OF ANIMALS WITH BACTERIAL PROTEOTOXINS (ANAPHYLATOXINS).*

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The discovery, a few years ago, by Friedberger (1) that powerful poisons were produced when active guinea pig serum was left together with emulsions of various bacteria, has had an important influence upon our conceptions of infectious disease, and, indeed, has furnished cause for reconsideration of the long accepted endotoxin theory of Pfeiffer. It has become at least a reasonable hypothesis that these toxic products of Friedberger, which he has, perhaps somewhat inaptly, named anaphylatoxins,[1] may be the sole factors in the toxemias of such diseases.

Our knowledge of these poisons is, of course, in many features incomplete. Their relation to the actual mechanism of serum anaphylaxis is not positively determined and, since this phase of the subject is quite distinct from the work reported in this paper, we may abstain from discussing it here. In the case of the bacterial anaphylatoxins more particularly, the work of the last few years has opened the important question as to whether or not the bacterial protein actually furnishes the matrix or substrate for the poison. This was the natural assumption of Friedberger and seemed also to follow from the earlier work of Vaughan (2), since there are so many distinct points of similarity between the complement-produced substances of the former and the toxic split products obtained with the aid of purely chemical treatment by the latter. This view is further supported by a number of workers by the apparently autolytic production of such poisons from bacteria in salt solution suspension.

However, the work of Keysser and Wassermann (3), in which anaphylatoxin-like poisons were produced in guinea pig serum when kaolin or barium sulphate was substituted for bacteria, seemed to contradict this conception, and of similar import are certain experiments of Bordet, and the recent work of Jobling and Petersen (4). The last named investigators especially have left little room for doubt that poisons apparently similar to those of Vaughan and

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[1] Although we have long hesitated further to complicate the nomenclature of this involved subject, we believe that much confusion can be avoided by substituting the term proteotoxins for all of the poisons of this class,—viz., poisons produced by the contact of active serum with bacteria, precipitates, kaolin, agar, starch, etc.

of Friedberger (Jobling and Petersen call them serotoxins) can be produced by serum enzymes from the proteins of the serum itself, when these have been exposed by the adsorption of anti-enzymes by the kaolin or by the bacteria.

It is somewhat uncertain, therefore, whether the poison engendered when bacteria and fresh serum are put together at 37.5° C. is a product of bacteriolysis, as at first supposed by Friedberger, or whether it is rather a product of the cleavage of other proteins, a process in which the bacteria play only an indirect part. It is impossible to settle this point at present, although we do not think that the occurrence of one process necessarily excludes the other.

However this may be, it has no direct influence upon the importance of poisons of this description in infectious disease. Whatever the matrix, the fact remains that they are produced when active serum constituents meet with bacteria in suitable quantitative proportions and at body temperature. Their actual occurrence when these elements meet within the animal body has been demonstrated experimentally by Friedberger and Nathan (5). We are, therefore, still justified in attributing to them an important part in bacterial toxemia, in accordance with the theory of Vaughan and the subsequent views of Friedberger.

This being the case, it is of great importance to our general conceptions of immunity to determine whether or not the animal body can develop increased resistance to these poisons. Our own studies were carried on in direct connection with work upon immunity in typhoid fever, a disease in which one of us in a previous paper (6) has attempted to show the great likelihood of participation of the anaphylatoxins.

In spite of a number of investigations bearing directly upon this point, we were unable to find a definite answer to this question, when, incidental to a general study of such poisons, we reviewed the literature.

As early as 1905, V. C. Vaughan, Jr., (7) attacked this problem in connection with the toxic split products obtained by the method of V. C. Vaughan, Sr., from the colon bacillus. His results justified him in concluding that "after the administration of several doses of gradually increasing strength, a point is reached at which the animal is able to withstand the injection of from two to three times the amount which would surely have proven fatal for an untreated control." He interpreted this rather as an evidence of acquired tolerance than as one of immunity in the ordinary sense of the word.

Bessau (8) later studied the same question by a number of different methods, both in relation with serum anaphylaxis and with bacterial anaphylatoxins directly. In one series of experiments he sensitized guinea pigs simultaneously with beef and with horse sera and, after the proper period of incubation, when full susceptibility was developed, he administered sublethal doses of one of these sera. After recovery, animals so treated were found to be less susceptible to reinjection with the other serum than were controls to which the latter serum only was given. These results obviously seem to indicate that the preliminary intoxication with the anaphylactic poison induced a non-specific tolerance.

Though indirectly of much interest, the importance of these experiments for the solution of the problem we are discussing rests upon the acceptation of the identity of the so called anaphylatoxins (proteotoxins) with the toxic sub-

stances involved in serum anaphylaxis,—an assumption which is indeed rendered likely by many observations, but which, after all, is not yet a matter of certainty. Of more direct bearing, therefore, are the other experiments of Bessau in which animals that had recovered from anaphylactic shock (both actively and passively prepared), were subsequently injected with typhoid anaphylatoxin (proteotoxin). Here, too, it was found that the animals receiving the preliminary treatment showed but slight symptoms and recovered from doses of the poison which killed the untreated controls. Bessau concludes from his experiments that anti-anaphylaxis is a condition based entirely upon this acquired and non-specific tolerance. We will discuss this view in our conclusions.

Ritz and Sachs (9) later claim to have observed that guinea pigs were protected against *B. prodigiosus* anaphylatoxin after they had been injected with a sublethal dose of the same substance, 20 to 45 minutes before.

Subsequently Friedberger and Lurà (10) repeated and contradicted the experiments of Bessau. In their conclusions they state that animals treated with sublethal doses of anaphylatoxin are just as susceptible as normal animals if reinjected after 24 hours with anaphylatoxin prepared either from homologous or from heterologous protein. They likewise obtained negative results when they treated serum sensitized animals with anaphylatoxin and subsequently reinjected the protein used for sensitization. The animals so treated remained as sensitive as the controls.

The available literature, though on the whole favoring the occurrence of such acquired tolerance to poisons of this description, is contradictory. Moreover, the few experiments cited by the investigators mentioned above, in which repeated injections of bacterial proteotoxin were given, are not sufficiently extensive to be convincing either affirmatively or negatively. For, as our protocols will show, there is frequently observed a marked normal difference in susceptibility to these substances by guinea pigs of the same weight and age,—a confusing factor which necessitates the study of large series before a conclusion can be reached.

METHODS.

The plan of our experiments was a simple one, aimed solely at determining whether guinea pigs treated with sublethal doses of anaphylatoxin, produced by putting together bacteria and fresh guinea pig serum, could be thereby rendered more resistant to subsequent injections of this poison.

The toxic substance was invariably prepared with typhoid bacilli in the following way: Typhoid bacillus, laboratory strain "J," was grown on agar slants for twenty-four hours and the growth was

taken up with one cubic centimeter of salt solution for each slant. This emulsion was then mixed with fresh guinea pig serum in proportions of one half agar slant to each four cubic centimeters of serum. The mixture was incubated for six hours and then centrifugalized for one to two hours. It should be stated that centrifugalization, however long continued, never completely freed the serum of bacteria. We tried subsequently to accomplish this by the addition of small amounts of inactivated, strongly agglutinating serum to the mixtures before centrifugalization, but even then a few microörganisms invariably remained in suspension.

Filtration through Berkefeld candles removed the bacteria but, strangely enough, also rendered the serum non-toxic. This is an observation which greatly surprised us, but which, as we later found, had also been made by Moreschi and Golgi (11). For these reasons it was not possible to work with an absolutely bacteria-free preparation. However, since our experiments deal with resistance to the acute effects of the poison itself, this fact does not in any way complicate our results.

Both the immunizing doses and the test injections were given intravenously, preliminary experiments with intraperitoneal immunization having been entirely negative. As will be seen, it appeared to us necessary to carry out a very large series of experiments before we ventured to draw conclusions. Every now and then guinea pigs are met with which show unusual susceptibility or unusual resistance to the poisons. One or two such animals in a series tend to upset confidence in the results and necessitate repetition. For these reasons also it was necessary to use almost as many controls as test animals. The controls were always heavier than the test guinea pigs, and controls were made both before and after the test guinea pigs were injected, in order to allow for possible alterations in toxicity during the period of incubation, which often exceeded one half hour.

EXPERIMENTS.

In tabulating the experiments we have purposely omitted recording the preliminary observations by which the toxicity of each particular poison, used for the first or immunizing dose, was determined. In giving the first injections we aimed to employ a dose

TABLE I.

Experiment 1.

An interval of 2 days was allowed to elapse between the first and the second injections.

No. of animal.	First weight, gm.	First dose.	Result.	Second weight, gm.	Second dose.	Result.
1	240	3.0 c.c.	Severe shock	235	3.5 c.c.	Death in 3½ min.
2	230	3.0 c.c.	Moderate shock	230	3.5 c.c.	Death in 2½ min.
3	235	3.0 c.c.	Moderate shock	230	3.4 c.c.	Death in 4 min.
4	225	3.25 c.c.	Moderate shock	220	2.5 c.c.	Death in 5 min.
Controls before experiment						
1				240	2.5 c.c.	Severe shock. Recovered.
2				230	3.5 c.c.	Death in 3 min.
Controls after experiment						
1				230	3.5 c.c.	Death in 3½ min.
2				225	3.5 c.c.	Very severe shock. Recovered.

In this experiment there is no evidence of increased resistance in the animals, if tested two days after the first administration of the poison.

TABLE II.

Experiment 2.

An interval of 4 days was allowed to elapse between the first and the second injections.

No. of animal.	First weight, gm.	First dose.	Result.	Second weight, gm.	Second dose.	Result.
1	225	3.25 c.c.	Very severe shock Recovery	225	3.5 c.c.	Death in 3½ min.
2	225	3.25 c.c.	Severe shock	225	3.5 c.c.	Death in 5 min.
3	200	3.25 c.c.	Moderate shock	200	3.5 c.c.	Death in 2½ min.
4	200	3.25 c.c.	Severe shock	200	2.5 c.c.	Death in 2¾ min.
Controls before experiment						
1				235	3.0 c.c.	Severe shock. Recovery.
2				240	3.5 c.c.	Death in 3½ min.
Controls after experiment						
1				225	3.5 c.c.	Death in 4 min.
2				190	3.5 c.c.	Severe shock. Recovery.

Here there is no evidence of increased tolerance if reinjection is practised after an interval of 4 days.

TABLE III.

Experiment 3.

An interval of 7 days was allowed to elapse between the first and the second injections.

No. of animal.	First weight, gm.	First dose.	Result.	Second weight, gm.	Second dose.	Result.
1	195	2.0 c.c.	Severe shock	200	3.5 c.c.	No shock.
2	210	2.5 c.c.	Severe shock	205	3.5 c.c.	No shock.
3	210	2.5 c.c.	Slight shock	185	3.0 c.c.	Very slight shock.
4	205	2.0 c.c.	Slight shock	200	3.0 c.c.	No shock.
5	195	2.0 c.c.	Slight shock	195	3.0 c.c.	Moderate shock. Recovery.
6	195	2.0 c.c.	Moderate shock	200	3.5 c.c.	No shock.
Controls before experiment						
1				210	3.0 c.c.	Death in 3½ min.
2				200	2.0 c.c.	Death in 5 min.
Controls after experiment						
1				235	2.5 c.c.	Death in 2¼ min.
2				240	2.5 c.c.	Death in 4 min.

In this experiment we obtained strong evidence that after 7 days a considerable degree of tolerance to the poison was established. The poison here used was exceptionally powerful and killed the heavier controls without exception in doses of 2 to 3 c.c., whereas the test animals, all of them lighter than the controls, lived, some of them showing no shock whatever.

TABLE IV.

Experiment 4.

An interval of 14 days was allowed to elapse between the first and the second injections.

No. of animal.	First weight, gm.	First dose.	Result.	Second weight, gm.	Second dose.	Result
1	220	3.0 c.c.	Severe shock	220	5.0 c.c.	Death in 3 min.
2	260	3.5 c.c.	Severe shock	217	4.0 c.c.	Slight shock. Lived.
3	250	3.5 c.c.	Severe shock	240	4.5 c.c.	Slight shock. Lived.
4	250	3.5 c.c.	Severe shock	230	4.0 c.c.	Slight shock. Lived.
Controls before experiment						
1				255	4.0 c.c.	Death in 2 min.
2				245	3.0 c.c.	Death in 2½ min.
3				235	2.5 c.c.	Death in 4 min.
Controls after experiment						
1				260	4.0 c.c.	Death in 3 min.
2				240	3.0 c.c.	Death in 3½ min.

Again we have evidence of the development of tolerance, as in the preceding experiment.

which would give moderate shock without killing the guinea pig. The controls mentioned in the tables are always those carried out with the poison used for the second injection, by which the resistance of the previously injected guinea pigs was tested.

TABLE V.

Experiment 5.

An interval of 15 days was allowed to elapse between the first and the second injections.

No. of animal.	First weight, gm.	First dose.	Result.	Second weight, gm.	Second dose.	Result.
1	185	2.5 c.c.	Severe shock		4.5 c.c.	Slight shock. Lived.
2	220	3.5 c.c.	Slight shock		4.5 c.c.	No shock. Lived.
3	225	3.0 c.c.	Moderate shock	Not	5.0 c.c.	No shock.
4	180	2.5 c.c.	Slight shock	taken for	4.5 c.c.	No shock.
5	185	2.5 c.c.	Slight shock	these	4.5 c.c.	Death in 4 min.
6	180	2.5 c.c.	Slight shock	seven	4.0 c.c.	No shock.
7	225	4.0 c.c.	Very severe shock	animals	5.0 c.c.	No shock.
Controls before experiment						
1				195	3.5 c.c.	Death in 2 min.
2				255	4.0 c.c.	Severe shock. Lived.
3				300	4.5 c.c.	Severe shock. Lived.
Controls after experiment						
4				235	2.5 c.c.	Death in 2 min.
5				240	2.5 c.c.	Death in 2½ min.
6				240	2.5 c.c.	Death in 3½ min.
7				210	2.5 c.c.	Death in 3 min.

This experiment, while supplying strong evidence in favor of the development of tolerance, illustrates most clearly the irregularity occasionally encountered in the reactions of normal guinea pigs. Note how five of the seven controls died of relatively small doses, in acute shock, whereas two slightly heavier animals, after severe shock, survived larger amounts. It is to be noted in this connection that small differences of weight often mean considerable differences of resistance, indicating probably that the younger guinea pigs are much more susceptible than the older ones, the difference being greater than could be accounted for by mere difference in weight.

The preceding protocols (tables I to V) sufficiently illustrate the methods by which our experiments were done. A complete tabulation of all our experiments, which include over seventy test animals and sixty controls, would needlessly lengthen our paper without materially adding to clearness. For this reason we will briefly summarize the results in tables VI and VII, adding explanatory re-

marks since it was not possible to include the weights of the individual animals and the dosage employed.

TABLE VI.

Resistance to the Poison of Animals Which Had Received One Previous Injection.

Second injection, after 2 to 5 days.		Controls.	
Total No. of animals.	No. of animals surviving.	Total No. of animals.	No. of animals surviving.
12	2	12	6

The dosage here was practically the same. The weight of the control animals averaged from 10 to 15 gm. more than that of the test animals at the second injection.

The results if analyzed in the individual experiments show that before the fifth day, as a rule, the guinea pig is not only more resistant or tolerant to the poison, but is still suffering from the effects of the first injection and rendered thereby even less resistant than normally.

TABLE VII.

Second injection, after 7 to 60 days.		Controls.	
Total No. of animals.	No. of animals surviving.	Total No. of animals.	No. of animals surviving.
45	24	35	7

The dosage here was often one and one half to twice as high in the test animals as in the controls. The controls in the individual experiments were always heavier than the test animals.

There can be no question, in these experiments, that a definite degree of increased resistance is present in the animals between the seventh and the sixtieth days. It should be noted that, in analyzing the individual protocols, the most uniformly positive results were obtained between the seventh and the fifteenth days.

We have records of a few animals in which the interval between the immunizing and the first injection exceeded sixty days, but in none of these has there been as consistent or reliable evidence of acquired tolerance as in the cases recorded above, in which the intervals were shorter.

SUMMARY.

Our experiments have shown definitely that guinea pigs, once injected with sublethal doses of bacterial proteotoxins (anaphylatoxins), acquire distinct tolerance to these poisons. The degree to which such resistance or tolerance is developed is never very high, in no case in our experiments exceeding the ability to withstand one and one half to twice the fatal dose of the poisons. During the three or four days immediately following the first injection the animals appear to be slightly less resistant than are normal controls, this depending probably upon the injury done by the administered poison. Tolerance begins to be evident after from four to seven days, seems to be most highly developed in about two weeks, but lasts in a diminishing degree for at least as long as sixty days.

Our experience, in this respect, with the poisons resulting from the contact of active serum and bacteria is similar to that of Vaughan with the toxic protein split products obtained by chemical methods.

The development of increased resistance definitely established, the questions immediately arise: (1) Is this tolerance specific? And (2) can it be passively transferred, with the serum, to a normal animal? We have begun to seek answers for these problems but as yet our data are too meager to permit definite conclusions.

The significance of the existence of higher resistance in animals treated with proteotoxins is far reaching both in connection with anaphylaxis and with immunity in general. We are not inclined to attribute to it as predominant a part in anti-anaphylaxis as is assigned to it by Bessau. For, in the first place, tolerance to the poisons is never developed to a very high degree, and, moreover, it does not become evident until three or four days after the first injection, while anti-anaphylaxis develops almost immediately after shock. However, there seems to us to be strong presumptive evidence that such tolerance to the poisons may play an important and, possibly, a non-specific part in anti-anaphylaxis, the chief underlying and specific cause of this phenomenon being the exhaustion of antibodies, or desensitization in the sense of Besredka.

The relation of such tolerance to the resistance of the animal to bacterial infection is, of course, obvious if we accept the possibility

of the production of such poisons in the injected body and their participation in the production of bacterial toxemia. We hope to throw more light on these relations in another paper dealing with the aggressin-like properties of the proteotoxins.

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EXPERIMENTS ON THE RÔLE OF LYMPHOID TISSUE IN THE RESISTANCE TO EXPERIMENTAL TUBERCULOSIS IN MICE.*

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In the reaction to tuberculous infection the part played by the endothelioid cell or endothelial leucocyte (Mallory) has received much attention in recent years. This is probably due to the early appearance of these cells in the process of formation of the tubercle and because of their well known phagocytic activities.[1] The other conspicuous element in tubercle formation, the lymphoid cell, probably because of its later appearance and its lack of phagocytic power, has been almost entirely neglected. The small round cell infiltration about the tubercle has indeed been regarded by most observers as of very secondary importance and is often referred to as resulting probably from tissue destruction.

In spite of the lack of interest among pathologists concerning the rôle of the lymphocyte in the tuberculous process, attention has been called by a number of clinicians to the prognostic importance of the lymphocytes in the circulating blood.[2] These observers have noted that in rapidly fatal miliary tuberculosis the lymphocytes fall, often to below 10 per cent. of the circulating white cells, whereas in patients with early, healed, or healing tuberculous lesions these cells are increased, sometimes forming more than 50 per cent. of the total leucocytes.[3] Moreover, it is well known that in acute rapidly fatal

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[1] For the literature see Goldmann, E. E., Neue Untersuchungen über die äussere und innere Sekretion des gesunden und kranken Organismus im Lichte der "vitalen Färbung," Tübingen, 1912, 52; and Evans, H. M., Bowman, F. B., and Winternitz, M. C., *Jour. Exper. Med.*, 1914, xix, 283.

[2] For the literature, see Brecke, A., in Brauer, L., Schröder, G., and Blumenfeld, F., Handbuch für Tuberkulose, Leipzig, 1914, i, 581.

[3] Wack, P., *Deutsch. Arch. f. klin. Med.*, 1914, cxv, 596.

miliary tuberculosis relatively few lymphocytes occur in the individual tubercles, while in the subacute form where a higher resistance of the individual may be assumed, the tubercles contain large numbers of lymphoid cells. These facts have a close analogy in the reaction of the polynuclear leucocytes in certain infections against which they are presumed to form the chief resisting factor.

LYMPHOCYTES AS FACTORS OF RESISTANCE.

Until recently, the round cell infiltration occurring about slowly growing or healing cancer, about cancer grafts in immunized animals, about failing tissue grafts in unsuitable or resistant animals, has been assigned, as in tuberculosis, a secondary rôle. The lymphoid elements here, too, are supposed to be present as a result of tissue disintegration. However, it has been shown that in the case of tissue grafts, the lymphocytes in all probability are the chief agents in causing the destruction of the introduced tissue. The chick embryo normally has no resistance against the growth of implanted tissues from a foreign species, and likewise shows a total absence of the round cell infiltration about the graft.[4] When, however, the chick embryo is provided with a graft of adult chicken lymphoid tissue, it becomes as resistant as the adult to the growth of implanted tissue from a foreign species, and, like the adult, shows an intense infiltration of small round cells about the foreign graft.[5] Furthermore, if the lymphoid system of an adult animal is depleted by means of X-ray, the animal loses its power of resistance to heterologous tissue and an implanted tissue from a foreign species will then grow readily. It is significant that in such animals there is a total absence of the round cell infiltration about the edges of the graft always present in resistant untreated animals.[6]

In the light of these results it seemed probable that the lymphoid cell might play a more important rôle in the resistance to certain infections than had previously been supposed. The fact of its presence in the reaction to tuberculous infections, its variation in the blood with the condition of the tuberculous individuals, and the recent ex-

[4] Murphy, Jas. B., *Jour. Exper. Med.*, 1913, xvii, 482.
[5] Murphy, Jas. B., *idem*, 1914, xix, 513.
[6] Murphy, Jas. B., *Jour. Am. Med. Assn.*, 1914, lxii, 1459.

periments of Lewis and Margot[7] suggested the advisability of investigating the part played by the lymphocyte in the resistance to tuberculosis. Lewis and Margot made the observation that rats and mice experimentally infected with tuberculosis developed large spleens. Splenectomized animals, however, lived longer after inoculation than normal animals. This anomalous result seemed difficult to explain. It is interesting to note in this connection that the inoculations were never made in less than two weeks after the splenectomy and were usually done in the third week. At this period the lymph glands and the lymphoid tissue of the body have shown considerable hypertrophy and many animals show a great increase over normal in the circulating lymphocytes.[8]

THE EFFECT OF X-RAY ON LYMPHOID TISSUE.

Heineke has shown that X-ray has an almost specific and immediate destructive action on the lymphoid system, and in small doses seems to have little, if any, effect on other cells of the body.[9] These results have been adequately confirmed by other observers. We have found that by carefully regulated doses of X-ray, repeated at intervals, a gradual atrophy of the lymphoid tissue may be accomplished without any appreciable effect on the other tissues, or on the general health of the animal.

TUBERCULOUS INFECTIONS IN X-RAYED MICE.

This specific effect of X-ray on the lymphoid tissue offers an excellent experimental method for testing the value of the lymphocyte in various conditions. Animals whose lymphoid tissue has been destroyed should be highly susceptible to those infections against which the lymphocytes play a part in the defense, while their resistance to those infections met by the polymorphonuclear leucocytes should be unaffected. With this idea in view the following experiments were planned.

[7] Lewis, P. A., and Margot, A. G., *Jour. Exper. Med.*, 1914, xix, 187.

[8] Dr. Linda B. Lange has made a series of differential counts on mice before and at intervals after splenectomy. These will probably be reported later.

[9] Heineke, H., *Mitt. a. d. Grenzgeb. d. Med. u. Chir.*, 1905, xiv, 21.

Experiment 1.—Fifty mice of about the same age and size were selected. These were divided into lots of ten each and subjected to the following treatment.

A. Ten mice were splenectomized and then given a daily 5-minute exposure to X-ray for 2 weeks. Average dosage less than ¼ unit (Holzknecht scale), average penetration No. 6, milliamperes 3–4. Spark gap 1 to 1½ inches.

B. Ten normal mice were given daily exposures to X-ray for 2 weeks in the same dosage as group A.

C. Ten mice were splenectomized on the date of inoculation 4 to 9 hours before this procedure.

D. Ten mice were splenectomized 8 to 10 days before the date of inoculation.

E. Ten normal animals served as controls.

All the animals were in good condition at the time of inoculation.

They were divided into small groups in order to prevent the spread of epidemics if any should develop, and were then inoculated with an emulsion of a 6 weeks' glycerin veal bouillon culture of bovine tubercle bacilli, each animal receiving a dose of 1 mg. of dry tubercle bacilli in 0.8 c.c. normal salt solution.[10] As control to the X-ray effect numerous mice have been given the same or larger doses and have shown no bad effects while under observation for several weeks afterward.

TABLE I.

Animals.	Average time of survival.	Percentage of animals with tubercle bacilli in various organs.					
		Exudate.	Spleen.	Liver.	Heart's blood.	Lungs.	Kidney.
Group A	7.3 dys.	100		100	40	80	100
Group B	7.0 dys.	100	100	100	60	80	100
Group C	9.1 dys.	100		100	50	80	100
Group D.....	19.7 dys.	100		100	90	100	100
Group E	18.5 dys.	100	100	100	80	70	100

Group A = 10 splenectomized mice given 14 daily exposures to X-ray. Group B = 10 normal mice given 14 daily exposures to X-ray. Group C = 10 mice splenectomized a few hours before inoculation. Group D = 10 mice splenectomized 8 to 10 days before inoculation. Group E = 10 normal mice as controls.

Table I shows the average number of days the various groups of animals lived and the percentage of animals showing tubercle bacilli in the blood, peritoneal exudate, and the various organs. The two X-ray groups, A and B, lived on an average about seven days after the inoculation, and group C, splenectomized a short time before inoculation, averaged only about nine days. Group D, the ani-

[10]This culture was kindly provided by Dr. Paul A. Lewis of the Henry Phipps Institute. It is termed by him Bovine C. The organisms were dried by pressing between filter papers, weighed, and then made into an emulsion by long grinding in normal salt solution.

mals splenectomized about ten days previous to the inoculation, averaged 19.7 days which is a little more than a day longer than the normal group E, which survived 18.5 days as an average. The widespread distribution and great number of organisms leave little doubt that the tubercle bacilli were the cause of death. To rule out epidemics of mouse typhoid, cultures were always taken from the heart's blood and the character of any organisms obtained was studied.

Experiment 2.—This experiment confirms and adds a further control to experiment 1. The groups of mice used were as follows:

A. Eight mice were splenectomized and given 12 daily exposures to X-ray in the same dosage as in experiment 1.

B. Nine normal mice were given the same X-ray exposures as those in group A.

C. Sixteen small normal mice were given 17 daily exposures of X-ray of the same intensity as those in groups A and B. The exposures were discontinued 4 weeks before the inoculations were made.

D. Ten mice were splenectomized 3½ weeks before inoculation.

E. Ten normal mice.

All these animals except 8 of group C were given 2 mg. of a 9 weeks' old culture of bovine tubercle bacilli of the same strain as that used in the first experiment. These animals were isolated in individual glass jars so as to prevent the spread of epidemics, should any develop.

TABLE II.

Animals.	Average time of survival.	Percentage of animals with tubercle bacilli in various organs.					
		Exudate.	Spleen.	Liver.	Heart's blood.	Lungs.	Kidney.
Group A.....	8.4 dys.	100		80	20	40	40
Group B.....	9.7 dys.	100	100	66	11	44	55
Group C.....	7.1 dys.	100	88	71	42	57	100
Group D.....	26.0 dys.	75		87	25	37	62
Group E.....	23.3 dys.	100	100	80	10	90	70

Group A = 8 mice splenectomized and given 12 daily exposures to X-ray. Group B = 9 normal mice given 12 daily exposures to X-ray. Group C = 16 mice given 17 daily exposures to X-ray; 8 of them were inoculated 4 weeks after X-ray was discontinued. Group D = 10 mice splenectomized 3½ weeks before inoculation. Group E = 10 normal mice.

Table II shows the death rate and distribution of the organisms. The age of the culture explains the longer survival of the animals even with twice the dose used in the first experiment. Groups A and

B, the splenectomized X-rayed and the normal X-rayed animals, lived an average of 8.4 and 9.7 days, respectively, after inoculation. Of the sixteen mice in Group C, the life of the eight which were inoculated averaged only 7.1 days after inoculation. Although these animals had had a month to regenerate their lymphoid tissue, they still showed a completely depressed resistance. As a matter of fact, the autopsies showed the spleens and lymph glands to be atrophic, with little sign of regeneration. The early death in these animals compared with the other X-rayed animals may perhaps be due to the fact that they were much smaller mice and had been given more frequent exposures to X-ray. Eight mice of this lot which were not inoculated but kept as a control to X-ray effect are still living and in perfect condition almost three months after X-ray treatment. The mice splenectomized three and a half weeks before inoculation, in agreement with the results of Lewis and Margot, lived longer than the normal animals.

The experiments were planned with the idea of testing the resistance of the animals to tuberculosis when the amount of the lymphoid tissue was varied. The X-rayed animals are at the bottom of the scale, having a system greatly depleted, and they were the first to die of the disease. Next come those splenectomized shortly before the inoculation; these may be considered as having a reduced amount of lymphoid tissue. The animals inoculated eight to ten days after splenectomy, having an active proliferation of the lymphoid cells in the glands and elsewhere, at this stage apparently have about the same resistance as the normal animals. The animals, however, splenectomized three or more weeks before inoculation outlive the normal animals by a number of days and probably represent an increased activity of the defensive agents.

DISCUSSION.

The question naturally arises: Do these treatments cause variations in factors other than the lymphocyte, which might play a part in the resistance to tuberculosis? X-ray in the amount used in these experiments does not affect the general health of the animal. The polymorphonuclear leucocytes are not decreased in number and may be increased. The X-rayed animals have a normal resistance to

certain infecting agents against which these cells form the defense and in some cases they may even have an increased resistance. The circulating large mononuclear cells are not appreciably affected. As evidence that the endothelioid cells are not destroyed, great numbers of these are found in the spleen and lymph glands after X-ray treatment, actively phagocyting the remains of the lymphocytes.[11]

The well known association of the lymphocytes with tuberculous lesions tends to support the conclusions indicated by these experiments. In the acute miliary type of the disease where it may be supposed that little resistance is being offered, the lymphocytes are relatively few in the tubercle, while in the subacute miliary tuberculosis these cells occur in masses about the lesion. As has already been mentioned, the lymphocytes in the blood fall in the rapidly advancing cases, while individuals with localized and well controlled lesions will show a marked increase in the circulating lymphocytes. It would seem, therefore, that these facts taken in conjunction with our experiments strongly suggest that the lymphocyte plays an important rôle in the animal's resistance to tuberculosis.

SUMMARY.

Mice either normal or splenectomized after exposure to X-ray are markedly more susceptible to bovine tuberculosis than are normal animals. Animals splenectomized a short time prior to inoculation are also more susceptible than normal, while those splenectomized eight to ten days before inoculation have about the same resistance as normal. The mice splenectomized three to four weeks before inoculation have a resistance increased over the normal, as has already been shown by Lewis and Margot. As X-ray in the doses used apparently affects only the lymphoid tissue and as the hypertrophy of the remaining lymphoid tissue after splenectomy is so rapid that the circulating lymphocytes may be much above the normal by the third week, it is concluded that this evidence, taken with the well known association of the lymphocytes with tuberculous lesions, points strongly to the lymphocyte as an important agent in the defensive mechanism against tuberculosis.

[11] Heineke, H., *loc. cit.* See illustrations.

ON THE CAUSE OF THE LOCALIZATION OF SECONDARY TUMORS AT POINTS OF INJURY.*

By F. S. JONES, V.M.D., and PEYTON ROUS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

Plates 18 to 20.

The localization of secondary tumors at points of injury has been so often remarked upon that it is unnecessary to cite specific instances. The cause for the phenomenon is unknown. Lubarsch[1] has shown that mouse tumors may be made to localize secondarily in the liver, about splinters implanted in this organ, but he did not attempt to explain the finding.

There are a number of observations which make it evident that the development of tumor metastases from tumor emboli is conditional upon a special set of circumstances. Schmidt[2] has shown that a large proportion of tumor cells cast off into the blood stream may die without giving rise to metastases. He found that the pulmonary arterioles of patients with visceral cancer often contain many tumor emboli that are dead or dying. The fact has been repeatedly noted that rats and mice inoculated intravenously with tumor fragments seldom develop growths in the lungs despite the fact that the same material causes tumors when implanted subcutaneously. So too in these animals the intraperitoneal inoculation of active tumor material yields comparatively few takes.

It has seemed to us possible to study some of the factors concerned in the secondary localization of tumors by means of experiments involving tumor localization on the lining of the peritoneal cavity. According to Schmidt, the intima of the blood vessels constitutes the essential barrier to invasion of the tissue by the cells of tumor emboli. The peritoneal lining presents much the same structural

* Received for publication, August 1, 1914.

[1] Lubarsch, O., *Med. Klinik*, 1912, viii, 1651.

[2] Schmidt, M. B., Die Verbreitungswege der Karzinome und die Beziehung generalisierter Sarkome zu den leukämischen Neubildungen, Jena, 1903.

features as the intima of the blood vessels, namely, a single layer of flattened cells covering a connective tissue. When tumor fragments are injected into the blood stream they are often surrounded by a thrombus and furthermore are so widely scattered that their fate is difficult to follow. Both these difficulties are avoided by the use of the peritoneal cavity.

The first question to present itself is that of the nature of the forces which prevent, or at least hinder, the take of tumor fragments inoculated intraperitoneally. Is there an immunity reaction in which the fluids of the cavity are concerned, or merely a resistance offered by an intact serosa? To test the point we have injured the peritoneum of mice by mechanical means, afterwards inoculating a tumor. For the purpose finely ground diatomaceous earth (*Kieselguhr*) has been used and an adenocarcinoma known in our laboratory as Mouse Tumor 33. Mouse Tumor 33 grows in discrete masses, seldom infiltrating or becoming diffuse (figure 1). Because of this it was especially suitable for the work.

Experiment 1.—Fifteen mice were injected intraperitoneally with 0.25 c.c. of finely ground, sterile *Kieselguhr* suspended in Ringer's solution, and three days later with 0.1 c.c. of a suspension of very small fragments of mouse carcinoma in Ringer's solution. Control animals were injected with the tumor suspension only.

At the end of two weeks nine of the mice receiving both *Kieselguhr* and carcinoma remained. They were killed and carefully autopsied. Seven had tumors in the subcutaneous tissue and on the peritoneum where the injecting needle had been thrust through. In all of these, nodules were found scattered throughout the peritoneal cavity, and in several the liver, spleen, and kidneys were involved. The remaining two animals were negative.

Eight controls survived the two weeks. Six had tumors along the track of the inoculating needle similar to those in the experimental animals, but in only one had dissemination taken place in the peritoneal cavity.

This experiment and others similar show that an acute injury to the peritoneum, mechanically caused, renders it more suitable for the lodgment and growth of mouse tumor. Microscopic examination of the nodules on the parietal peritoneum, in the mesentery, and on the surface of the liver and spleen of the experimental animals has revealed an interesting condition. The particles of *Kieselguhr* are not distributed evenly but lie in aggregates here and there in the midst of a layer of newly formed and very cellular connective tissue

covered with endothelium. The tumors are in general definitely localized to these areas (figure 2). Many small discrete clumps of neoplastic cells are to be noted lying embedded in the reactive tissue and covered with endothelium. In the case of the larger, more diffuse tumors the association with the *Kieselguhr* is also evident (figure 3).

Although the results are clear cut they do not enable one to conclude whether it is damage to the connective tissue or to the endothelium that renders the peritoneal surface susceptible. For the inoculations were made at a time when the *Kieselguhr* had but just cut its way through the endothelium. In a later series of experiments two weeks were allowed to elapse between the *Kieselguhr* inoculation and the injection of the tumor, in order that the endothelium might have opportunity for complete repair. Sections show that after this time the *Kieselguhr* is enclosed in small discrete accumulations of quiescent connective tissue, completely covered by endothelium.

Experiment 2.—Ten mice were injected intraperitoneally with 0.25 c.c. of finely ground *Kieselguhr* suspended in Ringer's fluid, followed two weeks later by 0.1 c.c. of a suspension of mouse carcinoma. Ten control animals received the tumor suspension only.

Five of the mice that received the *Kieselguhr* and carcinoma were alive two weeks after the injection of the latter. They were killed and examined at this time. In four, tumors had developed along the track of the injecting needle while in the remaining one the tumor had failed to take. In all four susceptible animals tumor nodules were found throughout the peritoneal cavity.

Autopsy of the seven surviving controls revealed the following: Four had growths along the track of the needle and in three the tumor had failed to take. In one of the susceptible animals a nodule was present in the mesentery directly opposite the point of injection. In the others the peritoneal lining was normal.

This experiment and others of the same sort show that an injured peritoneal lining remains favorable to tumor implantations after the endothelium has repaired itself completely. Experiments in which lycopodium spores were used as the foreign body have given identical results. Unlike the *Kieselguhr* the spores do not penetrate but are rapidly surrounded by endothelial cells and later encapsulated by connective tissue (Marchand). As a rule quite a number of them are found lying together in a web of newly formed connective tissue. The little nodules so composed are covered with endothelium (figure 4). They offer a most favorable locus for tumor im-

plantation (figure 5). We feel justified in concluding that it is the derangement of the connective tissue, rather than of the endothelium which renders an injured peritoneal lining favorable to the lodgment and growth of tumor fragments.

The damage caused by *Kieselguhr* or by lycopodium is punctate in character, but it is wide-spread and might conceivably alter the ability of the peritoneum to elaborate immune substances, or to form the medium of their passage. It has seemed necessary, therefore, to perform experiments involving a relatively insignificant and sharply localized damage to the peritoneum. One or several small, sterile glass rods, rounded at the ends, were introduced into the peritoneal cavity of mice through a trocar and followed later by a tumor suspension.

Experiment 3.—Two or three glass rods about 1 mm. in diameter and 8 mm. long were introduced into the peritoneal cavity of each of ten mice. Two weeks later the animals were inoculated intraperitoneally with 0.1 c.c. of a fine suspension of mouse carcinoma. Ten control animals also received the tumor material at this time.

Six of the experimental animals were alive after two weeks. Four had a tumor in the track of the inoculating needle. In all four, intraperitoneal tumors were found situated next to the glass rods and there only (figure 6). The remaining two mice were negative.

Three of the controls survived two weeks. Two had tumors in the injection track and the other was negative. In one of the susceptible animals a few tiny, discrete tumor nodules were found on the mesentery.

The injury caused by a smooth, glass rod where it lies in contact with the peritoneum renders this latter favorable to tumor implantation. An alternative explanation, that the localization of the neoplasm was due to an accumulation of tumor fragments in a dead space about the rods, fails, because there was no dead space, the rods being closely enveloped in mesentery.

Evidently the resistance manifested by a healthy peritoneum to the lodgment and growth of tumor fragments is not due to a general immunity reaction, but is referable to the physical characters of the lining membrane.

In the light of our results, one may ask whether the factor of injury may not play a part, heretofore unrecognized, in the peritoneal dissemination of certain visceral tumors of human beings. It is true that some growths are so malignant that fragments sown on an in-

tact serosa can successfully lodge and grow. This has been noted of certain rat and mouse tumors as well. But at the other end of the scale there are visceral growths which fail to localize on the peritoneal lining, although fragments of them must be distributed to it. With tumors of intermediate malignancy may it not be that the first fragments that are cast off die, and, causing inflammation, render the peritoneal lining more susceptible for future implants? To test the point mice were inoculated intraperitoneally with bits of killed tumor and later with particles of the living growth.

Experiment 4.—Fifteen mice were injected intraperitoneally with 1.0 c.c. of coarse particles of killed mouse carcinoma suspended in Ringer's fluid. The suspension had been heated in the water bath at 55° C. for 15 minutes, a temperature sufficient to kill the cells of the tumor. Three days later each animal received a second inoculation of 0.06 c.c. of a suspension of living tumor fragments. Fifteen control mice were also inoculated at this time.

Seven of the experimental animals were alive two weeks later. In all, tumors had developed in the inoculation track. In six, there were nodules throughout the abdominal cavity. In the remaining animal two discrete nodules were found in the mesentery.

Ten controls survived. Four were completely negative as regards tumor. Six showed tumors in the injection track. Three of these had one or two tiny, sharply circumscribed growths in the mesentery, and a fourth showed many disseminated growths. In the other two the peritoneal lining was healthy looking.

Jobling[3] has shown that the intraperitoneal injection of a suspension of rat carcinoma killed by heat may increase the susceptibility of animals for later subcutaneous implantations of the same tumor. The results of the present experiment might be referred to a similar hypersusceptibility, were it not that in other of our experiments with identical intraperitoneal findings there is no evidence for this, tumors developing along the track of the injecting needle in about the same proportion of control animals and those injected with the killed suspension. Furthermore, special tests have shown that killed suspensions of Mouse Tumor 33 do not induce hypersusceptibility.

Since dead tumor fragments in contact with the peritoneal lining render this latter more suitable for the lodgment and growth of tumor cells, it seems probable that the peritoneal dissemination of some human tumors may indeed come about through the death of the first tumor fragments cast off, and the reaction thus caused.

[3] Jobling, J. W., *Monographs of The Rockefeller Institute for Medical Research*, 1910, No. 1, 52.

In what way does an injury to the peritoneal lining, or, more precisely, to the subendothelial connective tissue favor tumor localization and growth? The observations of Schmidt,[4] already mentioned, offer a suggestion. Schmidt found that tumor cells which had lodged in the pulmonary arterioles were unable to penetrate the vascular endothelium directly, although they might proliferate and ramify within the lumen of the vessel. Whether they ultimately invaded the surrounding tissue depended upon whether they were supplied with a supporting stroma by the subendothelial connective tissue. Now, in the case of the peritoneal lining we have found that the reactive changes caused by an injury to the subendothelial connective tissue greatly favor the lodgment and growth of bits of tumor. It seems possible that the stroma for a tumor fragment might be elaborated with especial ease by a connective tissue in course of proliferation as the result of an injury. As bearing on the point, we have compared the growth *in vitro* of connective tissue reacting to the presence of a foreign body with the growth of normal connective tissue from the same region. Implantations were made into chicken plasma of bits of tissue from about glass rods embedded for various periods in the breast muscle of fowls. Fowls were chosen because they are extremely resistant to local infection and because their plasma can be readily handled.

Experiment 5.—Eight sterile, smooth glass rods about 1.5 by 8 mm. were inserted on successive days by means of a trocar into the pectoral muscles of each of three fowls. The rods were marked for identification. When they had been in place for 1, 2, 3, 4, 6, 8, 10, and 12 days, respectively, the fowls were killed and many small pieces of tissue from about the rods were implanted in chicken plasma and incubated at 41° C. No infection had occurred. Pieces of normal connective tissue, of fascia, and of muscle from near by were also implanted and incubated. The results were striking. No growth occurred from the control fragments or from those about rods that had been in place only one day. There was marked and very prompt emigration of large, rounded ameboid cells from the fragments that had been next to the rods for two or three days. In a few instances there was a definite growth of fibroblasts as well. The pieces removed from about rods that had been in place 4, 5, 6, and 8 days showed a profuse connective tissue growth which began after only a few hours of incubation. In fact, the rapidity and amount of this growth compared very favorably with that of some sarcomata and of embryonic tissue. By the twelfth day the tissue encapsulating the rods had become quiescent and little growth was obtained from it.

[4] Schmidt, M. B., *loc. cit.*

The experiment shows conclusively that connective tissue reacting to the injury caused by the presence of a foreign body has a proliferative energy greater than the normal. Furthermore, its growth in plasma takes place without that latent period which Carrel has described for normal adult connective tissue.[5] The rounded cells that emigrated from the tissue which had been two and three days in contact with the glass rods were doubtless wandering cells attracted by the foreign body; and the true growth that took place obviously came about by the proliferation of the many fibroblasts present in the reactive tissue. From such results it seems highly probable that connective tissue, reacting to an injury, is in a condition to elaborate the stroma for a tumor more rapidly and abundantly than normal tissue.

Altogether, the findings seem to us to indicate that the secondary localization of tumors at points of injury is referable to the presence at such points of a very cellular connective tissue which may come more readily than the normal to the support and nourishment of the tumor cells. A number of facts in the literature may be taken to support this view. To mention only two of them, Loeb and Sweek[6] have described epitheliomata of which the sluggish course was apparently referable to the resistance offered by an inert connective tissue; and Levin[7] has shown that the Flexner-Jobling rat tumor, inoculated into the normal testicle of rats and into testicles previously injected with *Scharlach R* and ether water, will grow only in the latter. Levin ascribes this finding to some chemical influence inducing a "precancerous state" in the testicle. It would seem more likely that it is referable to the presence of a highly labile connective tissue capable of immediate and active proliferation in support of the tumor. The rapid spread of tumor tissue in a wound is explicable on the same basis.

SUMMARY.

The cause of the frequent localization of secondary tumors at points of injury is not known. Our work deals with this problem.

[5] Loeb has found that regenerating kidney grows better than the healthy organ *in vitro* (Loeb, L., *Anat. Rec.*, 1912, vi, 109).

[6] Loeb, L., and Sweek, W. O., *Jour. Med. Research*, 1913, xxviii, 235.

[7] Levin, I., *Jour. Exper. Med.*, 1912, xv, 163.

For the experiments the peritoneal cavity has been employed as offering relatively uncomplicated conditions, and the fate of mouse tumor brought into contact with a peritoneal lining injured in various ways has been studied.

The injection of a suspension of mouse tumor into a healthy peritoneal cavity has little success as a rule compared with a similar injection into the subcutaneous tissue. We have found that the resistance of the peritoneal lining thus indicated can be largely if not completely abolished by the preliminary injection of a mechanical irritant (*Kieselguhr,* lycopodium). That the change thus brought about is independent of general immunity phenomena is shown by the fact that a local injury renders susceptible the part of the peritoneum immediately affected and that part only. Special tests show that the factor important in rendering the peritoneum more susceptible is the injury to the subendothelial connective tissue. Susceptibility persists after the endothelium has regenerated over the reacting connective tissue.

Schmidt has found that the cells of tumor emboli in the pulmonary arterioles are able to penetrate the endothelium of the vessel only after they have been provided with a stroma from the subendothelial connective tissue. Our findings are easily explained on the basis thus suggested. A connective tissue highly cellular and perhaps still proliferating as the result of injury may well elaborate the stroma for a tumor more rapidly than normal connective tissue. Tests of growth *in vitro* support this idea. Connective tissue reacting to an injury grows profusely and almost immediately when incubated in plasma, whereas normal tissue from the same region shows usually no growth whatever.

Dead tumor fragments in contact with the peritoneum cause a change favorable to the lodgment and growth of later tumor fragments. It seems not improbable that the peritoneal dissemination of certain human neoplasms may be accomplished indirectly through the death of the first tumor fragments cast off.

Our observations have been purposely confined to the effects of injury on the peritoneal lining ; but they seem to afford the basis for a generalization. The secondary localization of tumors at points of injury may be attributed with good reason to the presence at such

points of an active connective tissue capable of elaborating a stroma rapidly and abundantly. For it is the proliferation of the subendothelial connective tissue to form a supporting stroma that determines the fate of free tumor cells, whether these lie on the peritoneum or within a vessel.

EXPLANATION OF PLATES.

PLATE 18.

FIG. 1. A nodule of Mouse Tumor 33 on the serous coat of the intestine, showing the discrete, uninvasive character of the growth. The nodule is largely necrotic.

FIG. 2. A portion of the parietal peritoneum and abdominal muscle of a mouse receiving an injection of *Kieselguhr* and three days later one of mouse tumor. The animal was killed two weeks after the second injection. On the peritoneal surface are two nodules of reactive tissue containing *Kieselguhr* and a third such nodule in which the tumor has localized.

PLATE 19.

FIG. 3. A higher magnification of a portion of the tumor nodule shown in the preceding figure. The *Kieselguhr* is indicated by the arrows.

FIG. 4. Lycopodium spores on the surface of the spleen. They lie grouped together, are embedded in connective tissue, and covered by endothelium.

PLATE 20.

FIG. 5. Portion of a tumor associated with the reactive tissue about lycopodium spores. To be compared with figure 4. Part of the abdominal muscle is shown.

FIG. 6. Viscera of a mouse receiving an intraperitoneal injection of tumor fragments two weeks after the introduction into the peritoneal cavity of three small glass rods. There are discrete tumors (*a* and *b*) in the vicinity of the rods but none elsewhere.

FIG. 1.

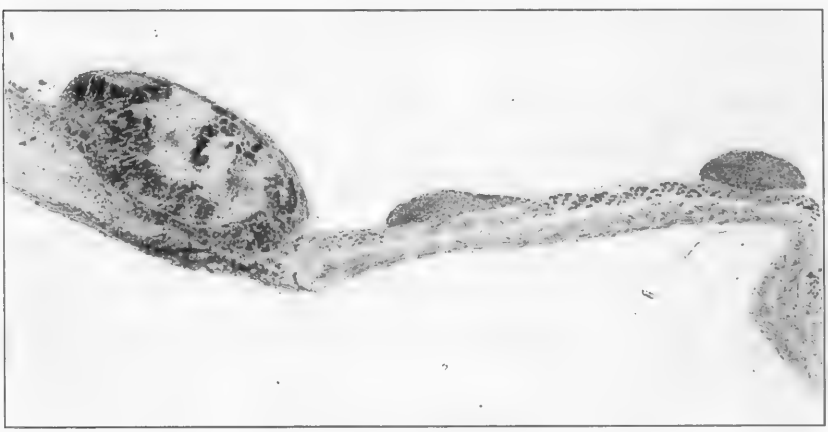

FIG. 2.

(Jones and Rous: Localization of Secondary Tumors.)

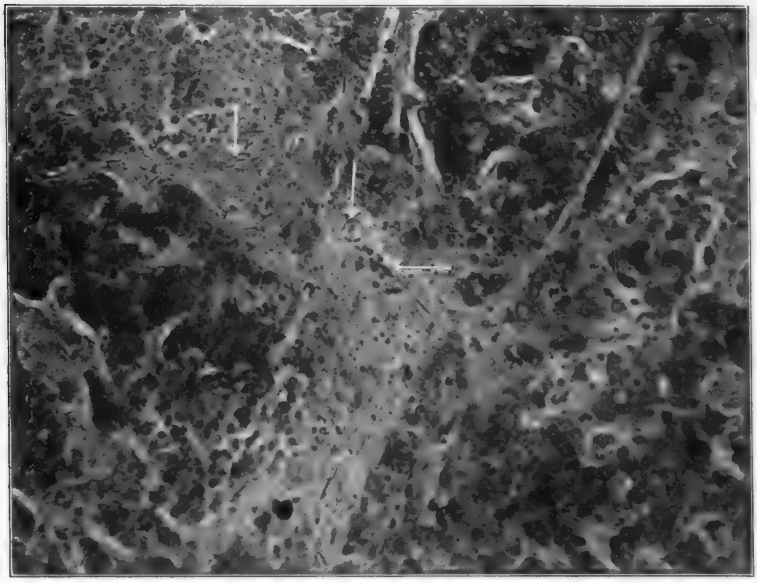

FIG. 3.

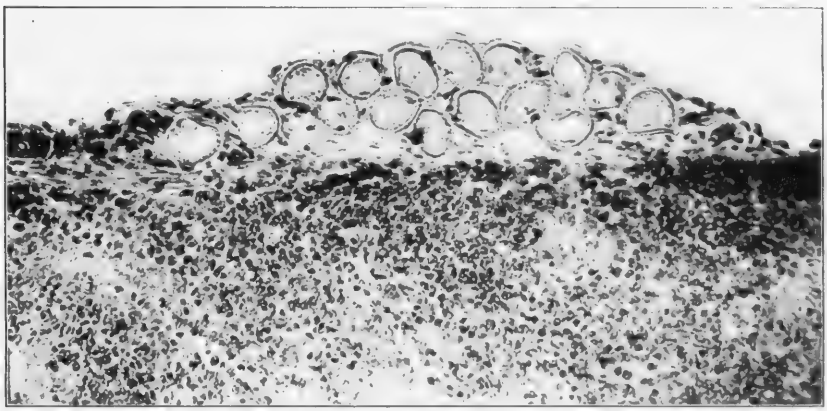

FIG. 4.

(Jones and Rous: Localization of Secondary Tumors.)

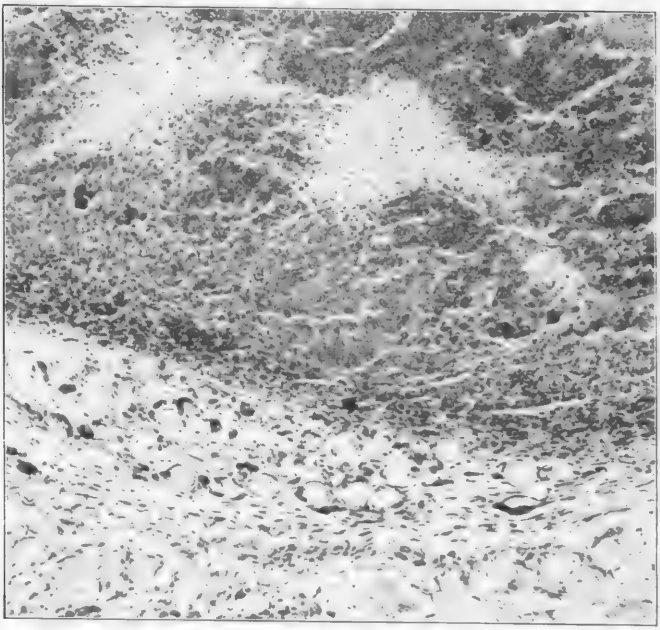

FIG. 5.

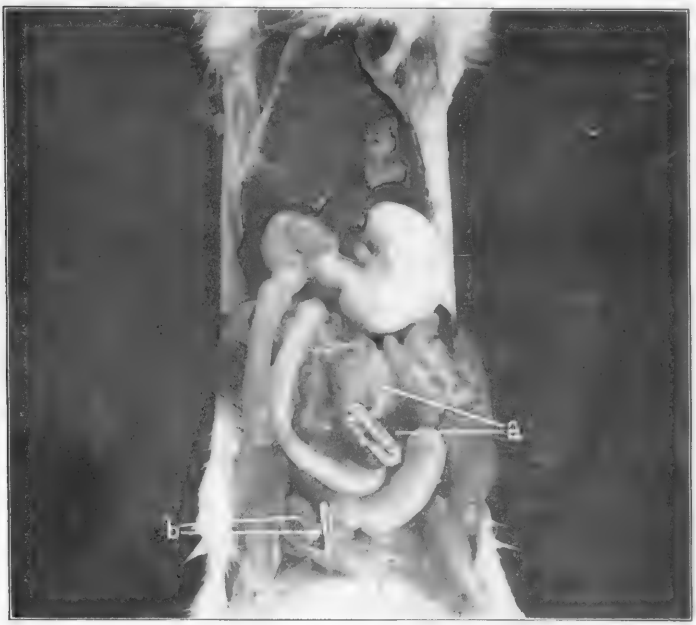

FIG. 6.

(Jones and Rous: Localization of Secondary Tumors.)

ON THE GREATER SUSCEPTIBILITY OF AN ALIEN VARIETY OF HOST TO AN AVIAN TUMOR.*

By PEYTON ROUS, M.D., AND LINDA B. LANGE, M.D.

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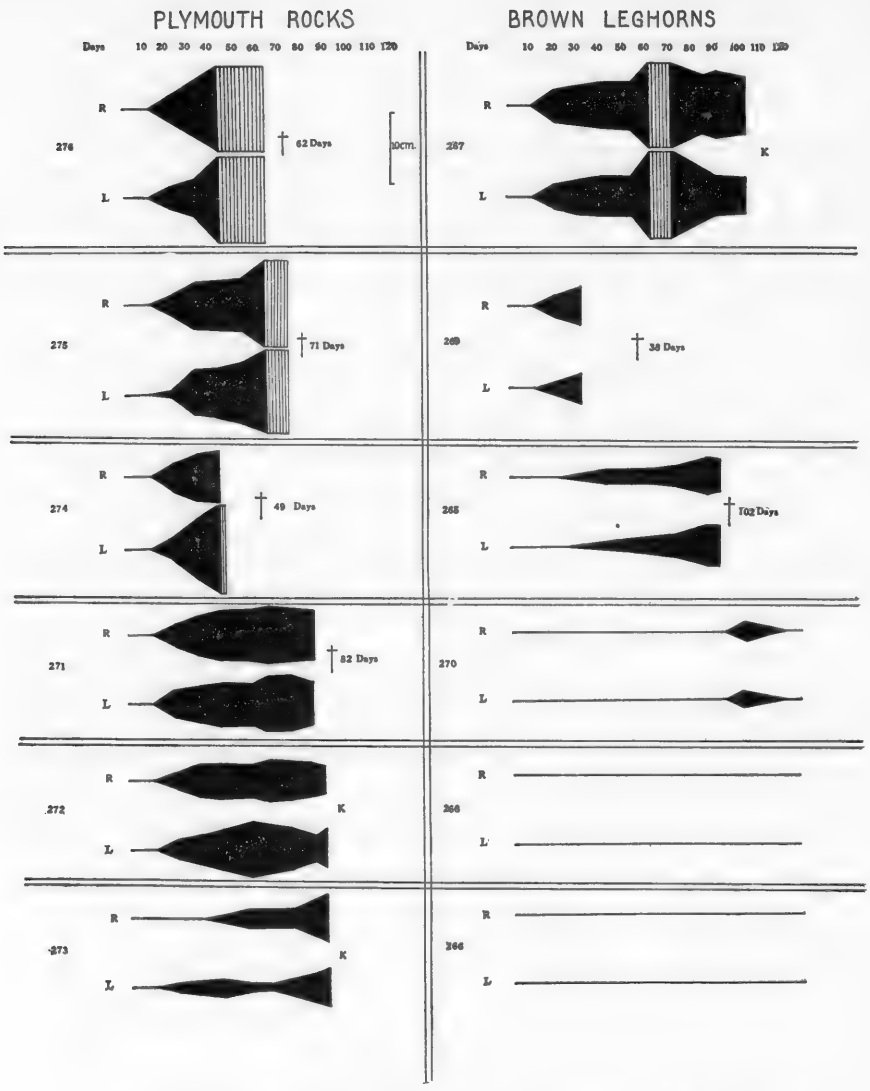
It has been the general experience of workers with transplantable tumors that they are most readily propagated in animals of the variety in which they appear as spontaneous growths (Jensen, Loeb). Some tumors are so specific in their demands as to grow only in individuals from certain sources and not in others of the same race. A spindle-celled sarcoma of the fowl studied in our laboratory was successfully transferred at first only to blood relations of the original host. In fowls of the same variety, but unrelated, it did not grow. The purpose of the present paper is to record an instance in which the behavior of a tumor was against the rule, its transplantation taking place more readily to hosts of an alien variety. Such instances are so rare as to deserve special report.

The growth in question, a tumor of the fowl (Chicken Tumor XVIII), has already been described at length.[1] It is a spindle-celled sarcoma curiously rifted with blood sinuses, and showing a tendency to metastasize to the voluntary muscles. It originated in a brown Leghorn hen, and was transferred to two successive series of such hosts, in which it grew slowly but with some increase in the percentage of takes. Of the third series of fowls inoculated several were barred Plymouth Rocks, and in these the tumor grew with especial rapidity. On subsequent inoculation to other barred Plymouth Rocks its growth was still more rapid. The findings seemed attributable either to an enhanced malignancy consequent on passage and independent of the host's variety, or to some special susceptibility of the Plymouth Rock breed. To test the matter a number of comparative inoculations were made.

* Received for publication, August 1, 1914.

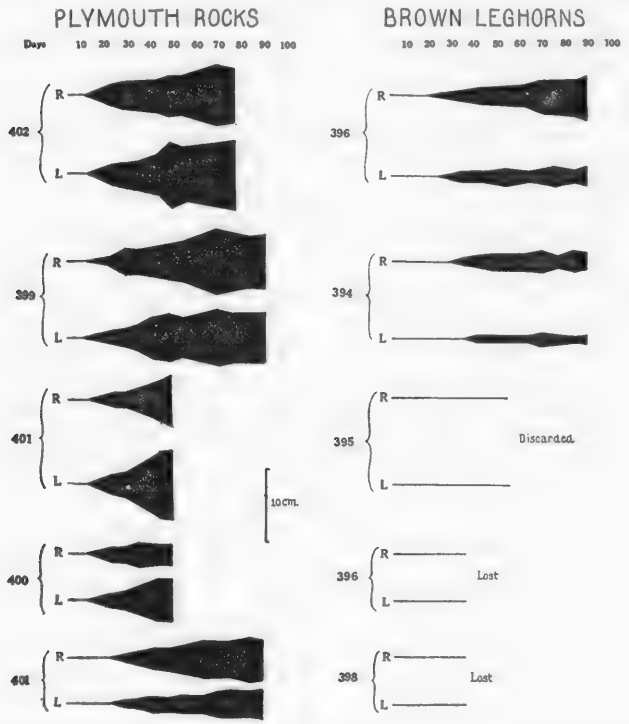
[1] Rous, P., and Lange, L. B., *Jour. Exper. Med.*, 1913, xviii, 651.



TEXT-FIG. 1. Results of the inoculation into Plymouth Rock and brown Leghorn fowls of bits of the rifted sarcoma taken from a Plymouth Rock. Two inoculations were made into each fowl. The time of appearance and rate of growth of the tumors are shown by diagrammatic outlines of which the width indicates the diameter of the tumor, and the length the period of growth. A hatched outline indicates that the tumor had filled the whole breast and could not be accurately measured. R and L = tumors of right and left breast. K = killed. The changes in size of the tumors appear abrupt, but this is because the measurements were taken at considerable intervals.

Experiment 1.—Small bits of fresh tumor tissue, of approximately equal size, from a barred Plymouth Rock fowl were implanted through a trocar, one in each breast of six brown Leghorn adults and six Plymouth Rocks. The results of the transplantation are shown in text-figure 1.

Experiment 2.—The tumor material was taken from a brown Leghorn inoculated in the preceding experiment. Implantations were made as before into each breast of five Plymouth Rock and five brown Leghorn fowls (text-figure 2).



TEXT-FIG. 2. Results of the inoculation into Plymouth Rock and brown Leghorn fowls of bits of the rifted sarcoma from a brown Leghorn fowl of the experiment recorded in text-figure 1.

Text-figure 1 shows that the sarcoma taken from a Plymouth Rock fowl and inoculated into Plymouth Rocks and brown Leghorns appeared in a smaller percentage of the latter and after a longer interval, and grew less well. This was also the case when

the material was derived from a brown Leghorn fowl (text-figure 2). Further observations have confirmed these findings. It may be urged that in the experiments figured, the results were due to an adaptive change in the tumor consequent upon its growth in several series of Plymouth Rocks, and not to be altered by sojourn in a single series of brown Leghorns. But as already mentioned, results entirely similar were obtained with the first Plymouth Rock fowls inoculated.

There are certain obvious physical differences between brown Leghorn and Plymouth Rock chickens to which the results might, conceivably, be due. The one breed is small and wiry, whereas the other is large and fat. Other things being equal, one might suppose that as hosts for all sorts of proliferating tissue the Plymouth Rocks would be better. To throw light on this point fowls of both varieties were inoculated with Chicken Tumor I, a simple spindle-celled sarcoma. This growth has already been mentioned for the striking specificity whereby its successful transplantation was at first confined to blood related Plymouth Rocks. It is now very malignant and grows well in chickens of many breeds.

Experiment 3.—Inoculations of Chicken Tumor I from a Plymouth Rock hen were made, as in experiments 1 and 2, into each breast of five Plymouth Rock and five brown Leghorn fowls. The results are given in text-figure 3.

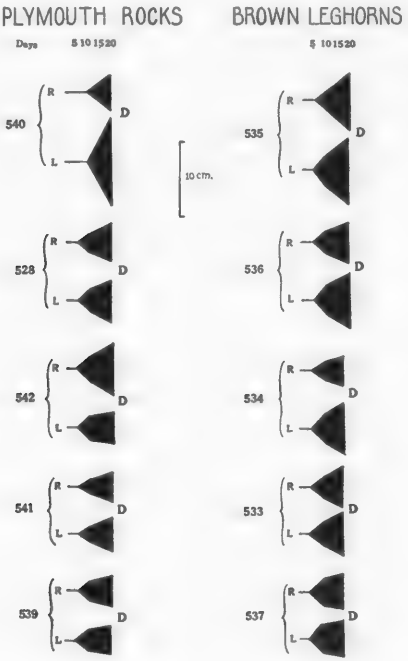
It will be seen (text-figure 3) that the tumor succeeded equally well in both varieties of fowls,—and this despite its early predilection for Plymouth Rocks. The findings with the rifted sarcoma can hardly be attributed then to gross physical differences in the chickens used.

Like our other transplantable chicken tumors the rifted sarcoma has a filterable agent as its cause. Yet in its transfer to new hosts, as carried out by introducing a bit of the fresh tumor tissue, a genuine transplantation is ordinarily involved, the new tumor arising by the survival and proliferation of the implanted fragment. The marked success of the sarcoma in hosts of an alien variety might conceivably be due either to an influence exerted on the transplanted cells or directly on the agent causing the disease. The results of a filtration experiment furnish some evidence for the latter view.[2]

[2] Rous, P., and Murphy, Jas. B., *Jour. Exper. Med.*, 1914, xix, 52.

Experiment 4.—Four Plymouth Rock fowls and four brown Leghorns were inoculated with equal portions of a Berkefeld filtrate prepared from the fresh tissue of the rifted sarcoma. A little sterile diatomaceous earth was added to the filtrate prior to its injection. The tumor used came from a Plymouth Rock fowl to which it had been transplanted after growing in two series of brown Leghorns.

The inoculated fowls were kept several months under observation. Two of the four Plymouth Rocks developed growths from which they eventually died. Of the four brown Leghorns only one developed a growth and this retrogressed.



TEXT-FIG. 3. Results of the inoculation of bits of the simple spindle-celled sarcoma into Plymouth Rock and brown Leghorn fowls. The tumor material was obtained from a Plymouth Rock.

No further experiments of the sort have been made because of the difficulty of obtaining the causative agent of the rifted sarcoma in active form. Unlike the agents causing our other chicken tumors it does not survive in the dried or glycerinated tumor tissue. Moreover, its activity in filtrates is very inconstant, and at best tumors are not produced until several months after the injection.

Among the numerous instances of the influence of race on the transfer of mammalian tumors we have been able to find but one in any way parallel to that here recorded. Tyzzer[3] transplanted a tumor of the Japanese mouse to hybrids of this breed and the ordinary white mouse, a variety completely insusceptible to the growth. In the F_1 generation of hybrids the tumor succeeded much better than in the Japanese mice. It may perhaps be remarked in this connection that Plymouth Rock fowls represent a mixture of several strains.

SUMMARY.

A transplantable sarcoma of the fowl, known as Chicken Tumor XVIII, in our series, succeeds better in chickens of an alien breed (Plymouth Rock) than in those of the variety in which it originated (brown Leghorn). This is not due to gross physical differences in the two breeds but to some more subtle factor and one which perhaps acts by influencing the agent causing the tumor. It would seem that Chicken Tumor XVIII, as it occurred in nature, was an instance of a disease appearing spontaneously in an animal of relatively insusceptible variety.

[3] Tyzzer, E. E., *Jour. Med. Research*, 1909, xxi, 519.

ON IMMUNITY TO TRANSPLANTABLE CHICKEN TUMORS.*

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The observation that the transplanted tumors of mice sometimes retrogress has been provocative of much research; for in it the phenomenon of acquired resistance to neoplasms was first clearly recognized. Now we know that this resistance is not peculiar to tumors but is elicited by non-neoplastic tissues as well. A resistance attributable to a causative element in mammalian new growths has still to be demonstrated, as, indeed, has such an element. Causative agents for transplantable chicken tumors, on the other hand, have been found. Those thus far studied are filterable. In the light of this fact a comparison of the phenomena of resistance to chicken tumors and resistance to mammalian growths becomes of much interest; since it may well be that there exist gross differences that would prove the two of different etiology. The present paper is concerned with such a comparison. In addition there will be taken up the question of the relationship between the agents causing different chicken tumors as indicated by the specificity of the resistance to them.

We have used for the work three distinct chicken tumors, namely, a simple, spindle-celled sarcoma (Chicken Tumor I), an osteochondrosarcoma (Chicken Tumor VII), and a spindle-celled sarcoma curiously fissured with blood sinuses and showing a tendency to metastasize to the skeletal muscles (Chicken Tumor XVIII). Most of the data have been obtained with Chicken Tumor I, which has been longest in our hands.

NATURAL RESISTANCE.

Natural resistance to the avian tumors will be briefly dealt with, since it has already been reported upon in describing the growths.

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Rat and mouse tumors, like the non-neoplastic tissues, can be successfully transferred under ordinary circumstances only to animals of the same species. This is true of chicken tumors as well. They will not grow in rats, mice, rabbits, or pigeons; and the spindle-celled sarcoma, the only one thus tested, will not grow in ducks. In fowls that are sick or emaciated the tumors do badly, either failing to develop after the inoculation, growing slowly, or retrogressing early. The same peculiarity has excited much attention in the case of mammalian growths. These latter grow best in young animals, and especially well in the new-born.[1] The influence of the age of the host upon chicken tumors has been tested only with the simple spindle-celled sarcoma. Young fowls have been found most susceptible as hosts for it, and in chick embryos it grows with extraordinary rapidity.[2]

Not a few mouse tumors are transplantable solely to animals of the variety in which the growth was spontaneous. A still greater specificity has been shown by Chicken Tumor I, which was transplantable at first only to blood relations of the original host and not to other varieties than the original until after months of propagation. The osteochondrosarcoma exhibits no preference for a special variety of fowl. The sarcoma rifted with blood sinuses shows what may be termed a reversed specificity, growing better in fowls of an alien sort (barred Plymouth Rock) than in the original brown Leghorn variety. This finding has been made the subject of a special paper.[3]

There exists an individual resistance to mammalian growths independent of all the factors thus far mentioned. Animals possessing it in its complete form fail to develop a tumor even though inoculated again and again. This is true of chicken tumors as well. But it is noteworthy in both cases that as the malignancy of the growth increases, owing to its sojourn in susceptible hosts, the number of animals insusceptible to it lessens. An individual naturally resistant to one form of mammalian tumor is frequently very susceptible to another. There is abundant evidence that this is true of avian growths as well.

[1] Unpublished work from this laboratory.

[2] Rous, P., and Murphy, Jas. B., *Jour. Am. Med. Assn.*, 1911, lvi, 741.

[3] Rous, P., and Lange, L. B., *Jour. Exper. Med.*, 1914, xx, 413.

Experiment 1.—Nine fowls were employed, four of them normal Plymouth Rocks, and the remainder brown Leghorns naturally resistant to the rifted sarcoma, as is shown by its failure to develop in them on a previous inoculation. All were now inoculated in the muscle of one leg with a bit of a slowly growing simple sarcoma (Chicken Tumor I), in the other with the rifted sarcoma. The inoculation of the simple sarcoma was unsuccessful and after seventeen days a second inoculation was made at the same site with more malignant material. The rifted sarcoma was then just beginning to appear. The final results are shown in text-figure 1.

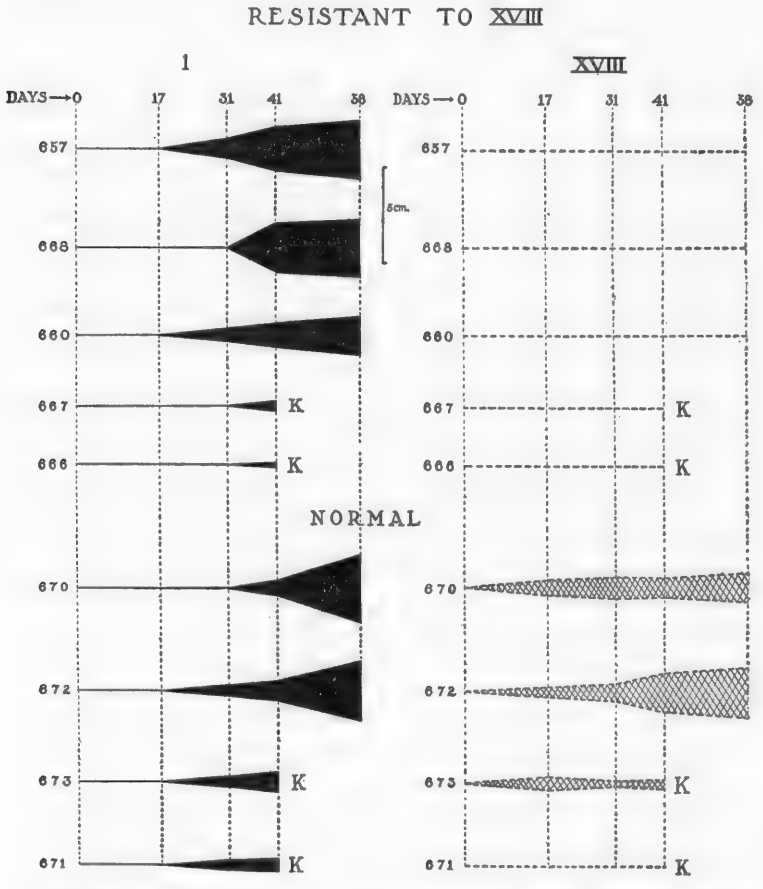
Text-figure 1 shows that fowls with a complete natural resistance to the rifted sarcoma and perhaps a slight acquired one (from the previous inoculation) were as susceptible to the simple sarcoma as normal fowls in which the rifted sarcoma grew well.

From all of the foregoing it is plain that the phenomena of natural resistance to chicken tumors are, in general, strikingly similar to those associated with rat and mouse tumors. The only apparent exception is in the tendency shown by the rifted sarcoma to grow better in fowls of an alien variety. Even here an instance somewhat similar may be found in mammals. Tyzzer bred together mice of two varieties, the one susceptible, the other insusceptible to a transplantable tumor of the Japanese waltzing mouse and found that the offspring of the F_1 generation were more susceptible than the susceptible parent. In the case of the rifted sarcoma the varieties of host tested were both the result of interbreeding several strains of fowls.

ACQUIRED RESISTANCE.

Some kinds of transplantable mammalian tumors grow progressively until the death of the animal; others after brief growth tend to become stationary and retrogress. The osteochondrosarcoma's behavior is of this latter sort. After a period of rapid enlargement as a chondrosarcoma in which spicules of bone gradually appear, it in most cases ceases to grow and is slowly absorbed. It not infrequently retrogresses after reaching a diameter of six or seven centimeters, but may take months to disappear, especially when it contains much bone. Only by the careful selection of tumors still growing has it been propagated. The simple spindle-celled sarcoma as a rule develops rapidly and progressively; but by the transplantation of slowly growing examples a retrogressing form may be obtained.

The rifted sarcoma develops slowly and with a considerable proportion of retrogressions.



TEXT-FIG. 1. Experiment 1. This shows that the simple sarcoma (Chicken Tumor I) implanted in fowls resistant to the rifted sarcoma (Chicken Tumor XVIII) grew as well as in normal fowls susceptible to the latter.

The time of appearance and rate of development of each tumor are shown by diagrams of which the width represents the diameter of the growth and the length its period of existence. A straight line indicates that no tumor developed. Cross-hatching indicates a rifted sarcoma, and solid black a simple sarcoma. The two are grouped in separate columns. The fowls are Nos. 657, 668, etc. K = killed.

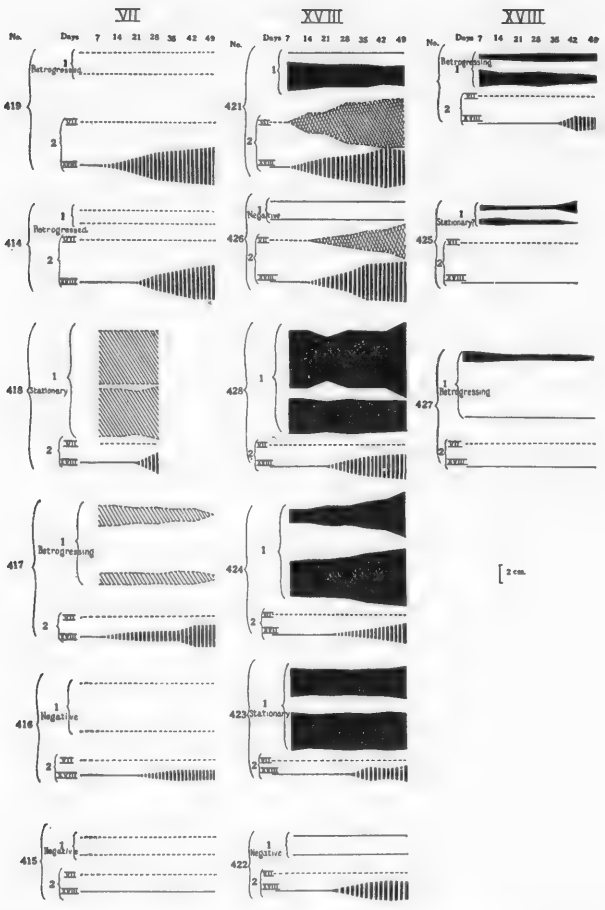
The amount of tumor material implanted has a marked influence on the course of all three chicken tumors. Retrogressing growths follow much more frequently the inoculation of single, small tumor bits than they do the inoculation of one to two cubic centimeters of the same tumor tissue, ground to a pulp. That dosage has an influence on the development and course of mammalian growths has long been known (Loeb, Clowes, and Baeslack).

Rats and mice in which tumors retrogress acquire resistance, as is shown by the fact that reinoculations within a few weeks usually fail of success. Some mouse tumors confer resistance on the host while they are still growing. This is especially true of tumors of retrogressing tendency. In the case of certain other neoplasms the factors which determine the course of the disease are so balanced that by mechanical means a stationary tumor may be made a growing one;[4] or reinoculations into a host in which the growth is stationary or retrogressing may be successful.

Exactly the same phenomena have been noted of the chicken tumors. The spindle-celled sarcoma grows rapidly and the success of secondary inoculations shows that it produces no notable concomitant resistance. The few individuals in which it is absorbed are usually resistant for a considerable time. The osteochondrosarcoma, a growth which tends to retrogress, produces a strong concomitant resistance (text-figures 2 and 5). Chickens in which it has been present for several weeks are always absolutely resistant on secondary implantation, and this at a period when the primary tumor is still growing. The slowly developing, rifted sarcoma often becomes stationary for long periods and then starts to grow again. By the use of malignant material hosts in which this growth is stationary or even slowly disappearing may sometimes be successfully reinoculated.

The resistance induced by the retrogression of a rat or mouse tumor is in part a pan-resistance but is most effectual against tumors of the same sort. Whether a pan-resistance to chicken tumors follows their retrogression has not been determined, but certainly much of the resistance is specific, as the following experiment shows.

[4] Loeb, L., *Jour. Med. Research,* 1901, vi, 28.



TEXT-FIG. 2. Experiment 2. This text-figure illustrates the fact that acquired resistance to the rifted sarcoma (Chicken Tumor XVIII) is slight as compared with that to the osteochondrosarcoma (Chicken Tumor VII); and it shows furthermore that resistance to the latter growth is to a large extent specific. All of the fowls (Nos. 419, 414, etc.) had been inoculated previously at two points. The character of this first inoculation (Chicken Tumor VII or Chicken Tumor XVIII) has determined the grouping in columns. For each fowl there are four diagrams representing tumor growth, or, in its absence, four lines. The diagrams bracketed together as 1 record the tumors of first inoculation; those bracketed as 2 record those of the second, the latter comprising an implantation with both Chicken Tumors VII and XVIII. The diagrams of the rifted sarcomata (Chicken Tumor XVIII) of first inoculation are given in solid black, those of the second in heavy hatching. A lightly hatched diagram indicates an osteochondrosarcoma of the first inoculation, and a cross-hatched one a tumor of the same sort following the second inoculation.

Experiment 2.—Six fowls previously inoculated with the osteochondrosarcoma and nine inoculated with the rifted sarcoma were chosen for this experiment. Some carried growths that were enlarging, some retrogressing growths, and others had shown themselves naturally resistant. All were now inoculated with the rifted sarcoma in the wing muscles of one side and with the osteochondrosarcoma at the same spot on the other side. 0.1 c.c. of a suspension of the fresh tumor tissue in Ringer's solution was used in each case. The course of the old tumors and the development of the new are shown in text-figure 2.

It will be seen from text-figure 2 that all of the fowls previously inoculated with the osteochondrosarcoma were now resistant to it. The malignancy of the material employed is proved by the rapidity with which it gave rise to tumors in two fowls previously implanted with the rifted sarcoma. This latter tumor grew in all but one of the fowls resistant to the osteochondrosarcoma. It also grew in seven of the nine hosts previously inoculated with a growth of its own sort. In one fowl the tumor of the first inoculation was actually retrogressing while that of the second enlarged.

When implanted simultaneously in the same host the chicken tumors preserve their character unchanged. The simple sarcoma metastasizes, as usual, to the lungs and other viscera, and the rifted sarcoma still gives secondary growths in the muscles, the source of each dissemination being clearly traceable from its histology. Sometimes one tumor grows rapidly whereas the others do badly or fail to grow (text-figure 4). So too it is with neoplasms of the rat and mouse. In a previous article the fact has been pointed out that the histological signs of resistance to these latter are identical with those to chicken tumors when allowance is made for the peculiarities of the two classes of host.[5]

Despite the efforts of many workers an immune principle effective against rat and mouse tumors has yet to be demonstrated in the blood of animals recovered from these growths. Crile and Beebe[6] succeeded in curing dogs of infectious lymphosarcoma by transfusing to them blood from other dogs in which the growth had retrogressed; but the lymphosarcoma has characters which distinguish it from the true neoplasms. Nevertheless, attempts to cure chicken tumors by means of transfusion have seemed advisable. Five fowls

[5] Rous, P., and Murphy, Jas. B., *Jour. Exper. Med.*, 1912, xv, 270.
[6] Crile, G. W., and Beebe, S. P., *Jour. Med. Research*, 1908, xviii, 385.

in which a relatively non-malignant form of the simple sarcoma was developing as the result of inoculation were bled from thirty-five to sixty-five cubic centimeters and an equal or slightly larger amount of blood was transfused to them from resistant fowls. In these latter the simple sarcoma had retrogressed and several intraperitoneal inoculations of sarcomatous tissue had from time to time been made without yielding tumors, a fact confirmed at autopsy. Transfusion was done at a time when resistance to the sarcoma may be supposed to have been at its greatest, that is to say, some two to three weeks after a massive injection of sarcomatous tissue. But in the fowls receiving the blood the tumors grew quite as well as in untransfused controls.

It is well known that not only does the retrogression of a mammalian tumor render the host unfavorable for subsequent tumor grafts but that injections of normal tissues, of normal blood even, will act to this end. Embryonal tissue is especially effective. In our experience the injection of hashed chick embryo does not confer resistance to the spindle-celled sarcoma of the fowl. But the tumor used was very malignant and may not have been sufficiently sensitive as an indicator.

Thus far the chicken tumors have been considered simply as transplantable new growths. The phenomena of acquired resistance to them resemble such as are seen under like conditions in the case of mammalian growths and suggest no more than these the presence of a causative agent distinct from the tumor cells.

RESISTANCE TO THE TUMOR-PRODUCING AGENTS.

By a special method there have been demonstrated two distinct forms of resistance against the simple sarcoma when it is transferred by grafting,—the one directed against the transplanted tumor cells, the other against the growth's causative agent.[7] Resistance of the latter sort will come into consideration in the findings now to be discussed.

With the exception of Königsfeld[8] workers with mammalian tumors have found that neoplastic tissue killed by drying fails to in-

[7] Rous, P., *Jour. Exper. Med.*, 1913, xviii, 416.

[8] Königsfeld, H., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1914, lxxiii, 316.

duce resistance against subsequent grafts. We have repeatedly attempted to induce with dried tissue resistance to the spindle-celled sarcoma of the fowl. The growth's causative agent remains active after drying, so it was necessary to make the first inoculations with material rid in some way of its tumor-producing property. The dried and powdered tissue was taken up in distilled water, heated at 60° C. for fifteen minutes, and injected intraperitoneally. For the later injections material submitted to 55°, 53°, or 50° C. for fifteen minutes, and finally unheated material, was used. Several groups of fowls were employed, but few came to the eventual test with the implanted growth, because nearly all developed tumors following the inoculation with unheated, dried tissue. Those remaining may well have been naturally resistant. If any protection is elicited by the injection of dried material in which the agent exists in attenuated form, it must certainly be very slight.

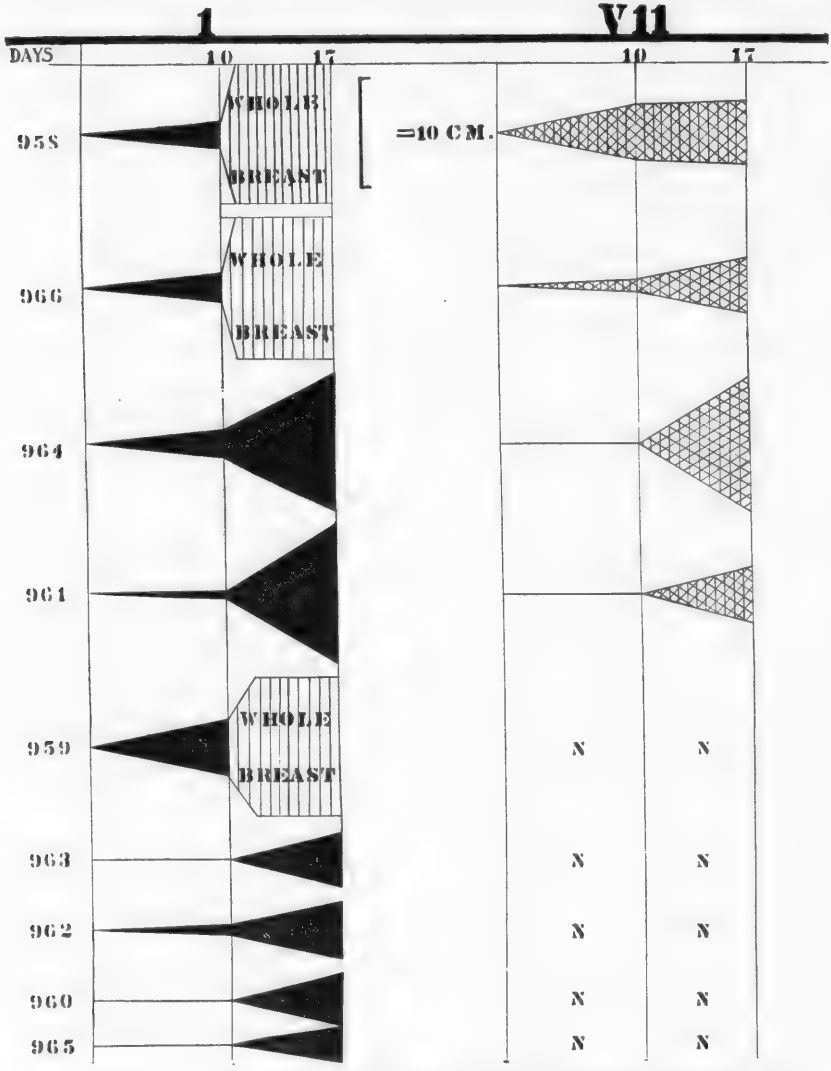
SPECIFICITY OF THE RESISTANCE.

That there exists a natural resistance to the agents is shown by their failure to produce tumors in some hosts. The question arises as to how far this resistance is specific.

Experiment 3.—Nine healthy Plymouth Rock fowls were inoculated, in one breast with 0.5 c.c. of a suspension of the dried tissue of an osteochondrosarcoma (Chicken Tumor VII), in the other breast with 0.1 c.c. of a like suspension of the dried spindle-celled sarcoma (Chicken Tumor I). The suspensions were made by rubbing up 1 gm. of dried tumor tissue in 9 c.c. of distilled water. The difference in dosage was to compensate for differences in the malignancy of the tumors. The results will be found in text-figure 3.

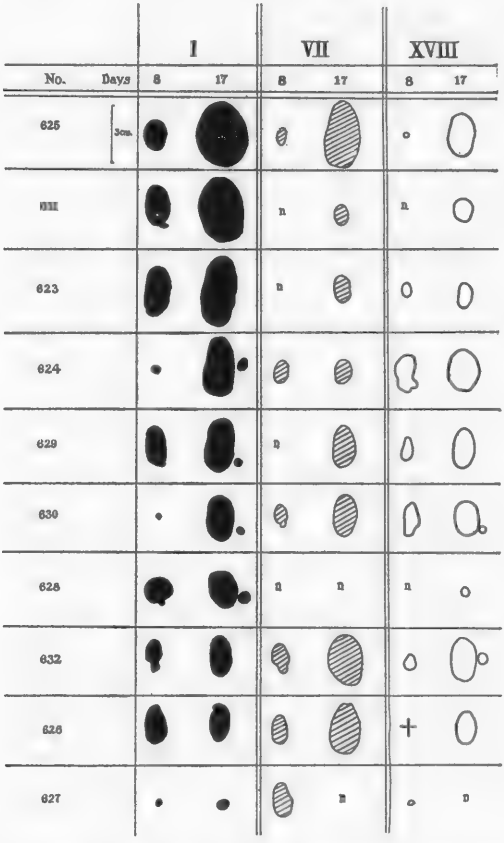
It is evident from text-figure 3 that the agents of the simple sarcoma and the osteochondrosarcoma are largely influenced by the same factors of natural resistance. In the experiment which it illustrates the period which elapsed before the appearance of a palpable tumor was so nearly the same for the two growths that the results can scarcely be referred to concomitant resistance induced by one tumor and effectual on the other.

With the rifted sarcoma a test of the above sort has not been possible because its agent is obtained apart from living cells only inconstantly and with difficulty. Comparative transplantation has been resorted to but this introduces a factor of error in that there



TEXT-FIG. 3. This shows that the same factors of natural resistance influence the activity of the agents causing two different chicken tumors. The fowls (Nos. 958, etc.) were inoculated in one breast with dried material of the simple sarcoma (Chicken Tumor I), in the other with that of the osteochondrosarcoma (Chicken Tumor VII). The diagrams are black for the simple sarcoma, cross-hatched for the osteochondrosarcoma.

are transferred with the agent tumor cells strange to the new hosts yet capable of active proliferation in many of them. With such a large disturbing element one would scarcely expect to learn much regarding the specificity of resistance to the agents. The following experiment gives evidence for the correctness of this view.

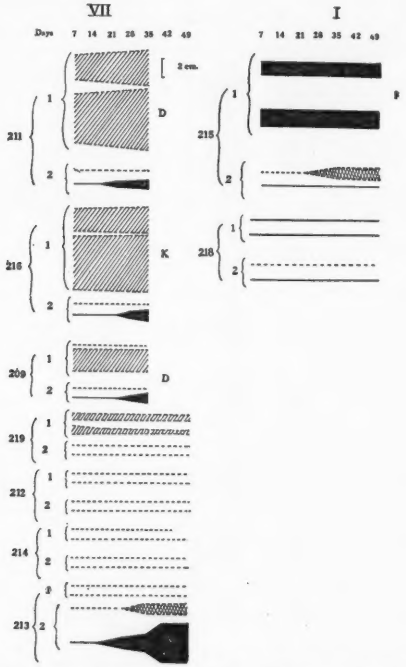


TEXT-FIG. 4. Fowls Nos. 625, etc., were inoculated simultaneously at different points with all three chicken tumors (I, VII, and XVIII). The text-figure shows that the growths varied independently of one another.

Experiment 4.—Ten healthy Plymouth Rock fowls were inoculated at different spots with all three chicken tumors (I, VII, and XVIII) in the amount of

0.1 c.c. of the finely ground fresh tissue. The sites chosen were in the muscle of both breasts, and the arrangement of the inoculations was varied from fowl to fowl. The results are shown in text-figure 4.

It will be seen from text-figure 4 that the tumors varied independently of one another. The findings as regards the simple sarcoma and the osteochondrosarcoma give no hint of the relationship seen in text-figure 3.



TEXT-FIG. 5. This text-figure has to do, like text-figure 2, with reinoculations; and the same general explanation holds good for it. The results of the first inoculation are given in the bracket 1 and those of the second in 2. The black and hatched diagrams are those of the simple sarcoma and the osteochondrosarcoma, respectively. The second inoculation was made with dried material of each growth. It will be seen that the agent of the simple sarcoma failed to give rise to tumors in fowls in which this growth had done badly on previous inoculation, whereas it caused growths in fowls resistant to the osteochondrosarcoma. The resistance against the latter tumor growth is also largely specific. Of seven fowls previously inoculated with it but one was susceptible on second inoculation. This fowl, No. 213, was supposed to be naturally resistant because of an unsuccessful inoculation some weeks previously, but the agents of both tumors engendered growths in it.

SPECIFICITY OF ACQUIRED RESISTANCE TO THE AGENTS.

Obviously the resistance acquired by a fowl in which a tumor has retrogressed must be effectual not only against the tumor cells but against the associated agent,—else this latter by acting on the cells of the host would produce a tumor. The following experiment indicates that acquired resistance to a tumor-producing agent is largely specific.

Experiment 5.—One fowl in which the simple sarcoma had retrogressed, one in which it was stationary, and four fowls carrying the osteochondrosarcoma were employed. They were inoculated, in one breast with 0.1 c.c. of a thin paste made by rubbing up dried tissue of the simple sarcoma with Ringer's solution, in the other with 0.5 c.c. of a similar paste of the dried osteochondrosarcoma.

As text-figure 5 shows, the four fowls carrying the osteochondrosarcoma evinced a complete resistance to it on secondary inoculation, whereas the simple sarcoma developed in three of them. The opposite result was obtained with the fowls in which the simple sarcoma had retrogressed or was stationary. Both now proved resistant to this growth, but in one the osteochondrosarcoma developed. The fact that the agent of the osteochondrosarcoma is relatively inactive renders the result more striking.

SUMMARY.

The phenomena of natural and acquired resistance to transplanted chicken tumors strikingly resemble those observed in the case of transplanted mammalian growths; and no more than those do they suggest that the tumors have an extrinsic cause.

That there may exist in fowls implanted with a chicken tumor a resistance directed against the tumor-causing agent distinct from the resistance manifested against the alien tumor cells has been shown in a previous article.[9] Both sorts of resistance are present in a fowl in which a tumor has retrogressed, the resistance in such an instance being acquired. That directed against the agent is largely specific, giving little if any protection against the agents

[9] Rous, P., *loc. cit.*

causing other tumors. There is some evidence that the conditions upon which a fowl's natural resistance depends are the same for the agents causing different chicken tumors.

It has proved impossible to protect chickens against the agent causing the simple sarcoma by injecting them with dried tumor material in which this agent has been attenuated by heat. The transfer of blood from resistant fowls to fowls with growing tumors is in our experience void of effect on the tumors.